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13. ABSTRACT ***(Maximum 200 Words)***

This project is designed to investigate intracellular signaling mechanisms associated with neuronal cell injury. In the acute form, this injury accounts for neural injury following stroke and head trauma, while in the chronic phenotype, it may account for degenerative diseases such as Parkinson's disease. Our preliminary studies have suggested that mitochondria play a pivotal role in the signaling processes that result in neuronal death. Accordingly, we have designed a series of experiments that are intended to elucidate the mechanisms by which mitochondria contribute to neuronal death, with the ultimate goal of identifying strategies for neuroprotection that can be applied to both acute and chronic disease states. In this project period we have completed a study that helps us to understand the impact of potential neurotoxins on mitochondrial function. We have essentially completed the construction of an imaging system for performing spatially and temporally resolved fluorescence imaging of mitochondrial function. We have also identified several novel features of mitochondrial function in neurons, including spontaneous depolarization of unknown origin. We have also begun the characterization of several new fluorescent probes for mitochondrial function. Thus, we have made solid progress in the initial period of this project.

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Mitochondrial Mechanisms of Neurotoxicity
DAMD17-98-1-8627
Ian J. Reynolds, Ph.D., Principal Investigator
Teresa G. Hastings, Ph.D., Co-P.I.

Introduction

This project is designed to investigate intracellular signaling mechanisms associated with neuronal cell injury. In the acute form, this injury accounts for neural injury following stroke and head trauma, while in the chronic phenotype, it may account for degenerative diseases such as Parkinson's disease. Our preliminary studies have suggested that mitochondria play a pivotal role in the signaling processes that result in neuronal death. Accordingly, we have designed a series of experiments that are intended to elucidate the mechanisms by which mitochondria contribute to neuronal death, with the ultimate goal of identifying strategies for neuroprotection that can be applied to both acute and chronic disease states. These studies are performed on cultured neurons and on tissue derived from mature rodents.

Progress Report.

The progress reported here relates to the revised statement of work dated 7/20/98. This SOW is now focused on the first two technical objectives of the original proposal, based on the recommendations provided by the review process. We have made significant progress on several of the goals outlined in the SOW, which are detailed below.

1) Microscope set-up. After a careful consideration of the available technology, we purchased a fluorescence imaging system based on an upright Olympus microscope (BWX50) that is served by a monochromator-based light source and that also includes computerized z-axis control and a computer controlled filter wheel in the emission light path. The acquisition is controlled using SimplePCI (Compix Inc), a software package with which we have considerable experience. This rig provides the maximum advantage in terms of both flexibility and temporal as well as spatial resolution. Thus, we are able to acquire and analyze highly resolved images of mitochondria, as well as to be able to perform both excitation and emission ratio imaging experiments with good time resolution. Many, although not all, of the images provided in this report were generated using this system.

2) Glutamate Injury Model. The first technical objective is concerned with the mechanisms underlying the injurious effects of glutamate in neuronal cultures. Several of the approaches that we have used required further refinement to address the issues raised in this technical objective. *Intramitochondrial Ca^{2+} determination.* Several methods have been reported for the estimation of $[Ca^{2+}]_m$ (e.g. (Peng et al., 1998; De Giorgi et al., 1996)). However, we estimated that the magnitude of the mitochondrial Ca^{2+} load would be enough to saturate any of the reporters that are currently available. Instead of directly measuring $[Ca^{2+}]_m$, we have tried to make a less direct but more quantitative approach by using the uncoupler FCCP to release Ca^{2+}, and then measuring

the magnitude of the Ca^{2+} release using a low-affinity Ca^{2+} indicator. As illustrated in figure 1 (top panel), the addition of FCCP some 5min after a glutamate stimulus produces a very large $[Ca^{2+}]_i$ transient. The magnitude of this transient is dependent on the concentration of the glutamate used in the initial stimulus (figure 1, middle panel), and is independent of extracellular Ca^{2+}. Interestingly, the mitochondrial calcium store is rather transient in nature (figure 5, bottom panel), so that the transient induced by FCCP is greatly diminished in magnitude within 10 min following the washout of the glutamate. We believe that the Ca^{2+} is released via the uniporter, because the FCCP-induced transient is not sensitive to cyclosporin A (which blocks transition) or to CGP 37157 (which blocks mitochondrial Na^+/Ca^{2+} exchange). Thus, although we still have some important work to do using this model, this appears to be a reasonable approach to estimate the $[Ca^{2+}]_m$, and will be valuable in our effort to link $[Ca^{2+}]_m$ to injury.

Measurements of $\Delta\psi m$. One of the current hypotheses that links mitochondrial Ca^{2+} accumulation to neuronal injury advocates a role for the permeability transition pore (PTP), a Ca^{2+} and oxidant activated pore in the inner mitochondrial membrane. Activation of this pore results in bioenergetic collapse. Our key indicator for pore activation is $\Delta\psi m$, because PTP activation will result in the loss of $\Delta\psi m$ which we can detect with a variety of voltage sensitive dyes (White, Reynolds, 1996). Accordingly, we have started to establish the protocol for measuring signals using JC-1, our preferred indicator of $\Delta\psi m$ in neurons using our new imaging system (Figure 2). With the greatly enhanced spatial and temporal resolution compared to previous studies we found marked spontaneous changes in $\Delta\psi m$ in unstimulated cultures. This unexpected phenomenon was most pronounced in the neuronal processes (figure 2, middle series of images). This has not previously been reported in any cells that we are aware of, and suggests that there is some intracellular signaling impacting on mitochondrial function. Given the centrality of the role of mitochondria in injury, understanding the normal function of mitochondria is clearly of great importance, so we are pursuing this observation further in order to learn more about the nature of the signaling process. Quantitation of this phenomenon presents something of a challenge. However, we have found that a running determination of the slope of the changes in the fluorescence signals helps to identify depolarization events (figure 2, lower panel).

Characterization of MitoTracker dyes. There is the potential for profound changes in mitochondrial shape and function in association with injury. Mitochondria may swell upon induction of PTP, although this has not yet been reported in intact, cultured neurons. There is also the potential for changes in the signal of $\Delta\psi m$-sensitive dyes that may not be directly related to $\Delta\psi m$ (Scanlon, Reynolds, 1998). Thus, the availability of fixable dyes that selectively label mitochondria could be a considerable advantage if they provide a robust and constant signal. For example, in our planned studies of the translocation of cytochrome c, having fixed, fluorescently labeled mitochondria for comparison to the immunohistochemical signal from cytochrome c would be immensely valuable. We evaluated a series of these dyes as potential tools for the labeling of mitochondria (figure 3), and investigated their properties in both neurons and astrocytes in primary culture. These dyes stain cells with a pattern characteristic of mitochondrion-selective labeling (figure 3, top panels). However, it is apparent that oxidants such as hydrogen peroxide, as well as $\Delta\psi m$ alterations with FCCP, have significant effects on the pattern of staining in a way that depends on the concentration of dye used to load the cells.

Thus, both FCCP (middle panel) and peroxide (lower panel) increase the dye signal of MitoTracker Green, and the staining pattern of both MitoTracker Orange and MitoTracker Red are similarly sensitive (data not shown). As recently described by another study (Scorrano et al., 1999), these dyes may also directly impact on either complex I or PTP. Thus, there are both advantages and limitations in the use of these dyes, and this process of characterization has been a very helpful enterprise which will enable us to use and interpret the use of the dyes appropriately.

3) Mechanism of "Death Factor" Release. The recent finding that mitochondria can release apoptosis-inducing factors such as cytochrome c and AIF (Liu et al., 1996; Zamzami et al., 1996) emphasize the need to understand the mechanism by which this release occurs. We have approached this issue in several different ways.

Characterization of PTP in Brain. We initially sought to compare the properties of PTP in brain to that of mitochondria obtained from liver. This is important because it has been suggested that PTP is the main trigger for the release of death factors from mitochondria. Mitochondria obtained from liver have been studied in great detail, and are the source of much of the understanding of the properties of PTP. Interestingly, there are some significant differences between brain and liver mitochondria in the characteristics of PTP. These findings are described in detail in a submitted manuscript (Berman, Watkins and Hastings).

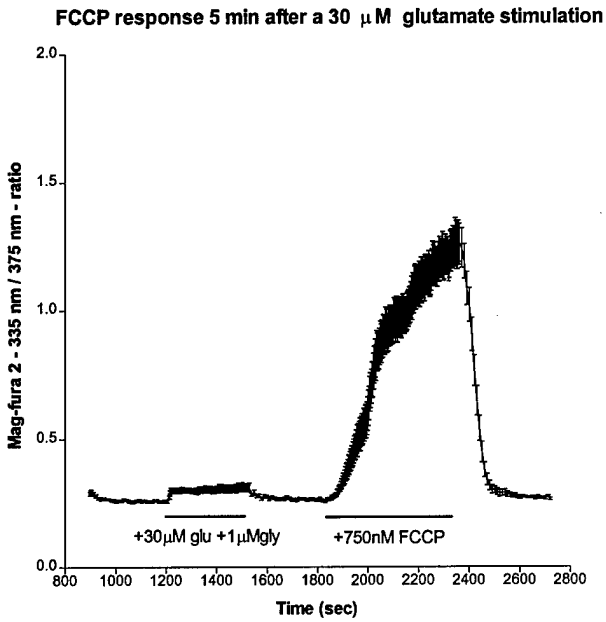
The Impact of Dopamine on Mitochondria. The release of death factors from mitochondria has been established as a critical event in many forms of apoptosis, but the mechanisms responsible for this release are not at all understood. We have previously established that dopamine produces apoptotic injury in neuronal culture (Hoyt et al., 1997), so we investigated the impact of dopamine and its oxidation products on mitochondrial function. Interestingly, dopamine and/or its oxidation products significantly altered mitochondrial respiration and could also induce PTP in brain mitochondria. The details of this study are provided in an accompanying reprint (Berman and Hastings, 1999).

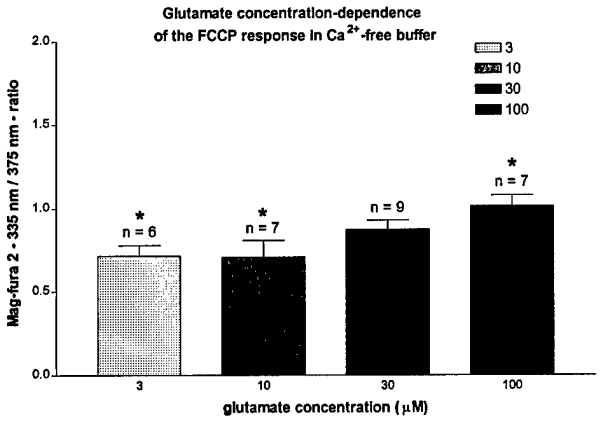
Tamoxifen Effects on Neurons. We initially proposed that tamoxifen might prove suitable for generating a mitochondrially-mediated form of apoptosis, based on observations by other laboratories (Ellerby et al., 1997). However, a subsequent report suggested a protective effect of much lower concentrations of tamoxifen (Custodio et al., 1998). We have investigated this in neurons, and found broadly similar results, in that low concentrations of tamoxifen offset the impact of glutamate on mitochondria, while high concentrations are quite toxic. However, tamoxifen did not protect neurons against toxicity triggered by glutamate. These findings are detailed in a submitted manuscript (Hoyt et al.).

In relation to this technical objective, we have also started to develop both Western blotting and immunocytochemical approaches to the quantification of cytochrome c release. These are challenging assays to perform quantitatively, and optimal assay conditions are still under development.

Figure 1. Approaches for estimating intramitochondrial calcium concentrations. (Top panel) Neurons loaded with the low-affinity calcium indicator magfura-2 are first exposed to glutamate with glycine to stimulate calcium entry. This results in mitochondrial calcium accumulation. Adding the uncoupler FCCP releases calcium from mitochondria, presumably via reversal of the calcium uniporter. The figure represents a typical trace and is the mean ± SEM of all of the cells in a single field of neurons. (Middle panel). Measurement of the peak of the response to FCCP shows a dependence on the concentration of glutamate used for the initial stimulus. Values of n represent the number of different coverslips used per glutamate concentration. (Lower panel) The mitochondrial calcium store is transient in nature. Neurons were exposed to FCCP at different times after the end of the glutamate stimulation. Note that the response to FCCP is almost abolished within 10 min of the glutamate stimulus, which presumably implies that the mitochondria have lost their calcium stores by that point. Each point represents the mean of the neurons from a single field.

Figure 1


FCCP response 5 min after a 30 μM glutamate stimulation
Mag-fura 2 - 335 nm / 375 nm - ratio
2.0
1.5
1.0
0.5
0.0
+30μM glu +1μMgly
+750nM FCCP
800 1000 1200 1400 1600 1800 2000 2200 2400 2600 2800
Time (sec)


Glutamate concentration-dependence
of the FCCP response in Ca2+-free buffer
3
10
30
100
Mag-fura 2 - 335 nm / 375 nm - ratio
2.0
1.5
1.0
0.5
0.0
* n = 6
* n = 7
n = 9
* n = 7
3
10
30
100
glutamate concentration (μM)

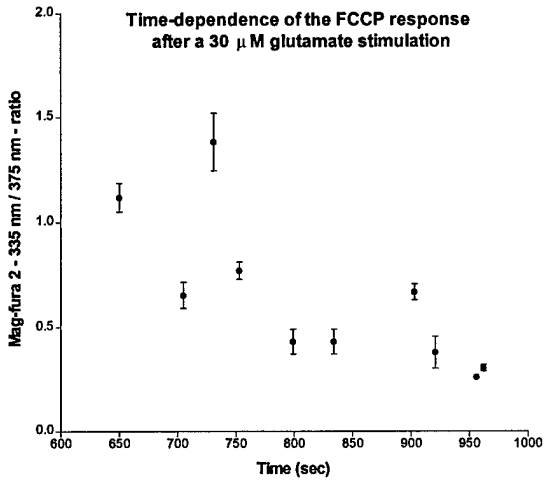
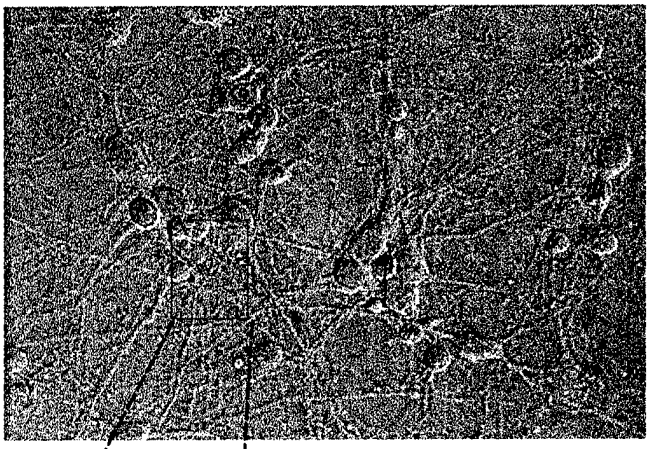

Time-dependence of the FCCP response
after a 30 μM glutamate stimulation
Mag-fura 2 - 335 nm / 375 nm - ratio
2.0
1.5
1.0
0.5
0.0
600 650 700 750 800 850 900 950 1000
Time (sec)

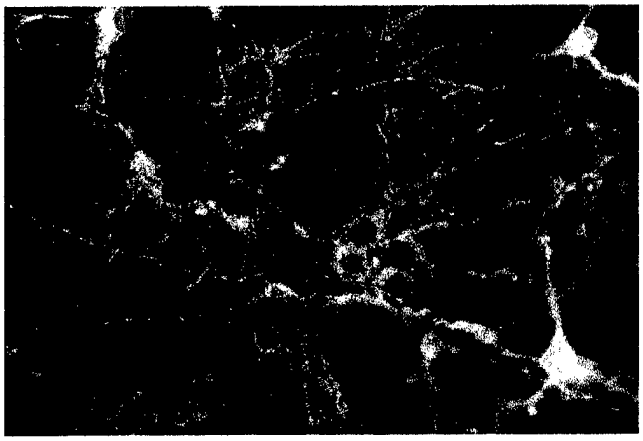
Figure 2. Spontaneous changes in $\Delta\psi m$. The top left panel shows a brightfield image that corresponds to the fluorescent JC-1 image on the top right. Note that the processes are brightly stained, while the cell bodies are less distinct. (Middle panels). This series of images shows changes in the brightness of a single process from the field. These images were taken over the course of a 45min experiment. Not that the indicated process starts out relatively dim, presumably reflecting hyperpolarized mitochondria, and then becomes progressively brighter, then more dim and then very bright. These represent spontaneous changes in signal. (Bottom panel). We are currently devising methods to detect and quantitate these events. This panel shows the raw fluorescence intensity of a single region of interest from a single process in red. We then calculated the slope of the change in intensity by measuring a four point slope of data smoothed using a three point moving average. Upward deflections represent mitochondrial depolarizations. The green marks represent event detected using the criteria we established. We continue to refine these criteria and this analysis method.

Figure 2

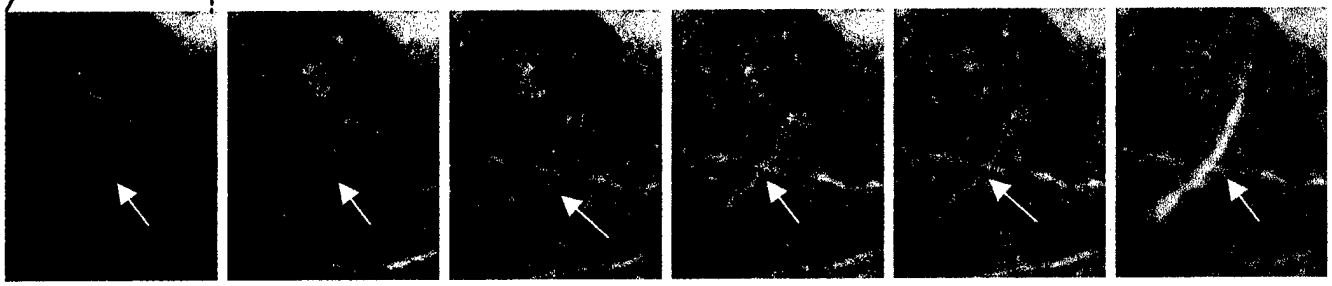
Phase Contrast Micrograph of Primary Cortical Neurons



JC-1 Fluorescence of Corresponding Field



Series of Fluorescent Images Showing Signal Spike Over Time in Neuronal Cell Process



Region of Interest Signal Measurements and Event Marks Over Time

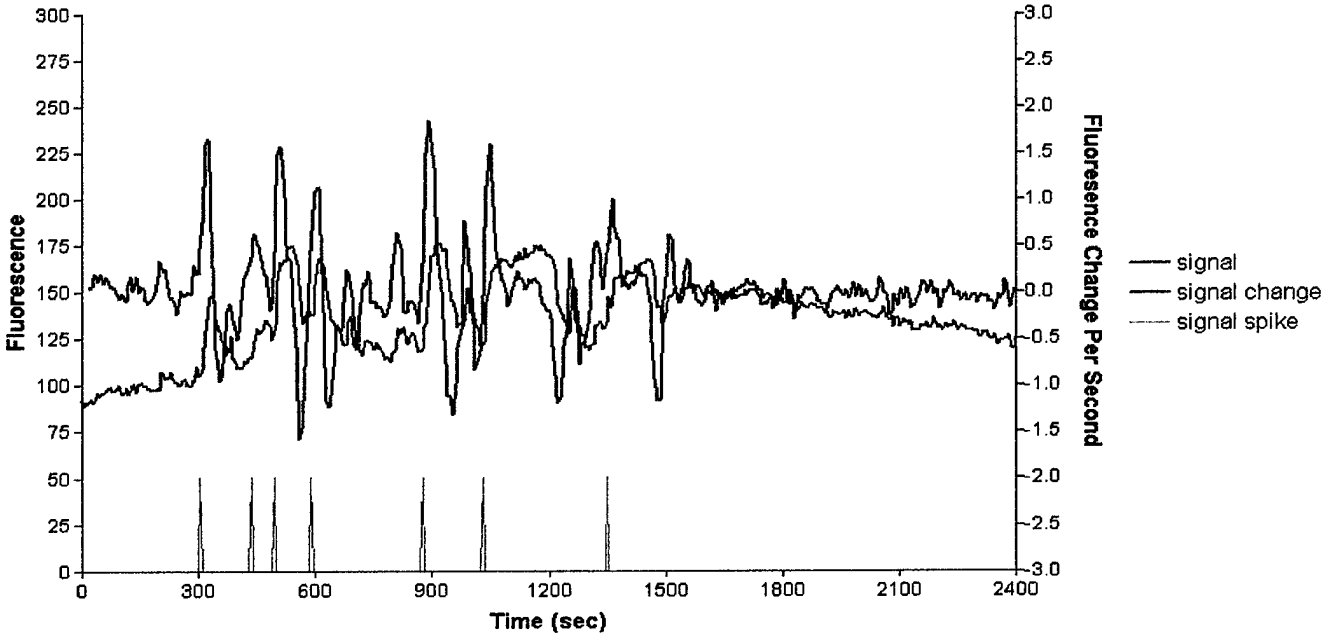
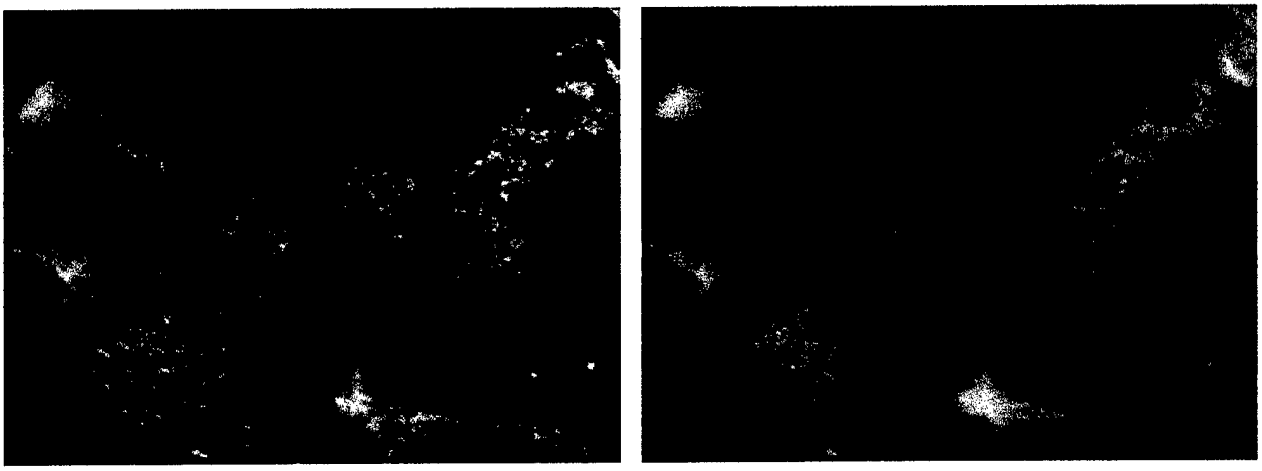


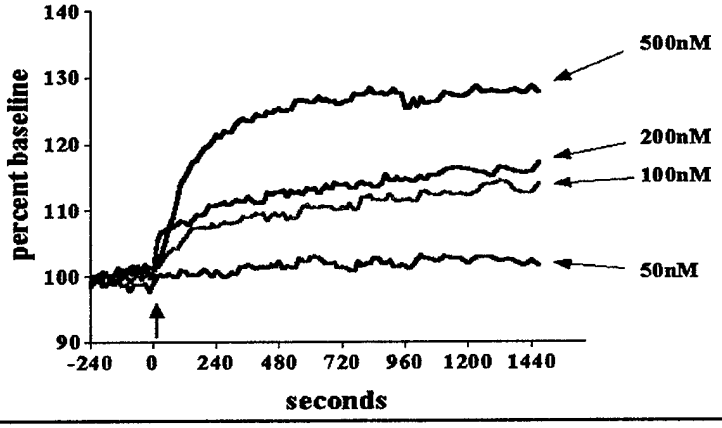
Figure 3. Imaging mitochondria with MitoTracker dyes. The top panels represent a field of astrocytes loaded with MitoTracker green before (left) and after (right) exposure to hydrogen peroxide. Not that the signal is initially quite punctate, consistent with a mitochondrial localization of the dye. The relocalization of the dye was not expected, and suggests that the staining might not be as invariant as advertised. (Middle panel) FCCP also changes the intensity of the dye signal when it is added to dye loaded cells, but in a way that depends on the dye concentration used to load the cells. Each of these traces is the mean of a field of astrocytes, and is representative of at least three cells per condition. (Lower panel) When astrocytes are pretreated with peroxide the intensity of staining increases when the cells are subsequently loaded with dye. These bars represent mean data from at least three different coverslips per condition.

Figure 3

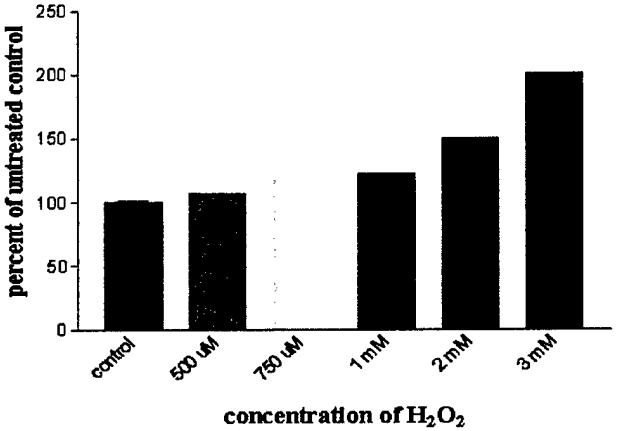
Pattern of MitoTracker Green fluorescence in astrocytes prior to and following treatment with H_2O_2



Response of increasing concentrations of MitoTracker Green to treatment with 750nM FCCP



Intensity of MitoTracker Green fluorescence in astrocytes pretreated with increasing concentrations of H_2O_2



Key Research Accomplishments.

- Completed a comparison of permeability transition in brain and liver mitochondria
- Completed a study of the impact of dopamine and metabolites on the function of brain mitochondria
- Completed a study on the impact of tamoxifen on mitochondrial function
- Developed novel assay for estimation of mitochondrial calcium accumulation
- Discovered existence of spontaneous mitochondrial depolarization in neuronal cultures
- Provided a detailed characterization of the properties of MitoTracker dyes in neural cells

Reportable Outcomes.

All of the papers and abstracts cited here are included in the appendix.

The following paper has been published:

Berman, S.B. and Hastings, T.G. Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria: implications for Parkinson's disease. J.Neurochem. 73:1127-1137 (1999).

The following paper is in press:

Reynolds, I.J and Hastings, T.G. The role of the permeability transition in glutamate-mediated neuronal injury. In: Mitochondria and pathogenesis, Lemasters, J.J. and Nieminen, A.-L. (Eds), Plenum Press, New York. (1999).

The following papers have been submitted:

Berman, S.B., Watkins, S.C. and Hastings, T.G. Isolated brain mitochondria do not readily undergo permeability transition: biochemical and structural analysis.

Hoyt, K.R., McLaughlin, B.A., Higgins, D.S. and Reynolds, I.J. Inhibition of glutamate-induced mitochondrial depolarization by tamoxifen in cultured neurons.

The following abstracts have been published.

Scanlon, J.M. and Reynolds, I.J. Effect of ubiquinone analogues on glutamate induced injury in rat forebrain neurons. Society for Neuroscience, **25**:2131 (1999).

Buckman, J.F., Pal, S.K., Kress, G.J. and Reynolds, I.J. Mitotracker dyes as markers of mitochondrial volume. Society for Neuroscience, **25**:2131 (1999).

Conclusions.

These initial studies represent a solid start for the work proposed in this project. As originally indicated in the proposal, there is an element of assay development that is necessary for the successful completion of this project. The construction of the new imaging system was an important step in the development of the novel assays, and the interesting observations on the spontaneous changes in mitochondrial membrane potential underscore the value of this new system. We have also made significant advances in our ability to measure calcium transport by mitochondria in intact neurons, and also to study the impact of factors that alter mitochondrial function.

These experiments help to provide a more complete and accurate picture of the operation of mitochondria in neurons, both under physiological and pathological conditions. Mitochondria obviously have a critical role in normal cell function in generating ATP from glucose. Understanding the ways in which it is possible to interrupt the pathological processes in which mitochondria participate without altering the normal physiological function will be essential if mitochondria are to represent a viable therapeutic target. We feel that the initial findings reported here will help to provide those necessary insights.

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Liu X, Kim CN, Yang J, Jemmerson R, Wang X (1996) Induction of apoptosis in cell-free extracts: Requirement for dATP and cytochrome c. Cell 86:147-157.

Peng TI, Jou MJ, Sheu S-S, Greenamyre JT (1998) Vizualization of NMDA receptor-induced

mitochondrial calcium accumulation in striatal neurons. Exp.Neurol. 149:1-12.

Scanlon JM, Reynolds IJ (1998) Effects of oxidants and glutamate receptor activation on mitochondrial membrane potential in rat forebrain neurons. J.Neurochem. 71:2392-2401.

Scorrano L, Petronilli V, Colonna R, Di Lisa F, Bernardi P (1999) Chloromethyltetramethylrosamine (MitoTracker Orange™) induces the mitochondrial permeability transition and inhibits respiratory complex I: implications for the mechanism of cytochrome c release. J.Biol.Chem. 274:24657-24663.

White RJ, Reynolds IJ (1996) Mitochondrial depolarization in glutamate-stimulated neurons: An early signal specific to excitotoxin exposure. J.Neurosci. 16:5688-5697.

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APPENDIX

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Ian J. Reynolds, Ph.D.

Contents

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Dopamine Oxidation Alters Mitochondrial Respiration and Induces Permeability Transition in Brain Mitochondria: Implications for Parkinson's Disease

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*Departments of *Neuroscience and †Neurology, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A.*

Abstract: Both reactive dopamine metabolites and mitochondrial dysfunction have been implicated in the neurodegeneration of Parkinson's disease. Dopamine metabolites, dopamine quinone and reactive oxygen species, can directly alter protein function by oxidative modifications, and several mitochondrial proteins may be targets of this oxidative damage. In this study, we examined, using isolated brain mitochondria, whether dopamine oxidation products alter mitochondrial function. We found that exposure to dopamine quinone caused a large increase in mitochondrial resting state 4 respiration. This effect was prevented by GSH but not superoxide dismutase and catalase. In contrast, exposure to dopamine and monoamine oxidase-generated hydrogen peroxide resulted in a decrease in active state 3 respiration. This inhibition was prevented by both pargyline and catalase. We also examined the effects of dopamine oxidation products on the opening of the mitochondrial permeability transition pore, which has been implicated in neuronal cell death. Dopamine oxidation to dopamine quinone caused a significant increase in swelling of brain and liver mitochondria. This was inhibited by both the pore inhibitor cyclosporin A and GSH, suggesting that swelling was due to pore opening and related to dopamine quinone formation. In contrast, dopamine and endogenous monoamine oxidase had no effect on mitochondrial swelling. These findings suggest that mitochondrial dysfunction induced by products of dopamine oxidation may be involved in neurodegenerative conditions such as Parkinson's disease and methamphetamine-induced neurotoxicity. **Key Words:** Dopamine—Mitochondria—Permeability transition—Parkinson's disease—Quinone—Respiration.
J. Neurochem. **73,** 1127–1137 (1999).

In Parkinson's disease (PD), the cause of the degeneration of dopaminergic neurons of the substantia nigra is unknown, but evidence suggests that oxidative stress is involved (for review, see Fahn and Cohen, 1992). One source of oxidative stress that is unique to dopaminergic neurons is the presence of dopamine (DA) itself, as DA can form reactive oxygen species (ROS) and quinones through two different pathways. First, DA is metabolized via monoamine oxidase (MAO) to produce hydrogen peroxide (H_2O_2) and dihydroxyphenylacetic acid (Maker et al., 1981). H_2O_2, if not reduced by cellular antioxidant mechanisms such as GSH and GSH peroxidase, can react with transition metals such as iron to form hydroxyl radical (Halliwell, 1992). This molecule will immediately react with lipids, DNA, and susceptible amino acids in proteins, thus causing cellular damage (Halliwell, 1992). Second, the catechol ring of DA can undergo oxidation to form DA quinone and ROS such as H_2O_2 and superoxide anion ($O_2^{\bullet -}$) in a reaction that can occur either spontaneously in the presence of transition metals or enzymatically (Graham, 1978; Hastings, 1995). The DA quinone is electron-deficient and reacts readily with cellular nucleophiles such as sulfhydryl groups on free cysteine, GSH, and cysteinyl residues in proteins (Tse et al., 1976; Graham, 1978). The reaction between the DA quinone and sulfhydryl groups leads to covalent modification of protein and free thiols, forming cysteinyl–DA conjugates (Tse et al., 1976; Graham, 1978; Fornstedt et al., 1990; Hastings and Zigmond, 1994). Because free thiols are important antioxidants in cells and protein cysteinyl residues often play critical roles in protein function, alterations of either free or protein thiols could lead to cellular toxicity.

DA is known to be toxic both in vitro (Graham, 1978; Michel and Hefti, 1990) and in vivo (Filloux and Townsend, 1993; Hastings et al., 1996), and we have shown that the formation of cysteinyl–DA conjugates

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Preliminary reports of these findings were presented at the 27th Annual Meeting of the Society for Neuroscience, New Orleans, LA, U.S.A., October 25–30, 1997.

Abbreviations used: CsA, cyclosporin A; DA, dopamine; H_2O_2, hydrogen peroxide; MAO, monoamine oxidase; $O_2^{\bullet -}$, superoxide anion; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; PTP, permeability transition pore; ROS, reactive oxygen species; SOD, superoxide dismutase; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

correlates with DA-induced neurotoxicity (Hastings et al., 1996). In addition, we have shown that DA oxidation products can inhibit the function of specific proteins, the DA and glutamate transporters (Berman et al., 1996; Berman and Hastings, 1997), and others have recently reported similar effects on the activities of tryptophan hydroxylase (Kuhn and Arthur, 1998) and tyrosine hydroxylase (Xu et al., 1998).

The protein targets that are critical to the toxicity induced by DA are not yet known, but likely candidates include many of the proteins important in mitochondrial processes. The critical role of mitochondria for cellular survival is well known, and mitochondrial dysfunction has recently been elucidated as an essential target in the induction of apoptosis as well as in excitotoxic neuronal death (Deckwerth and Johnson, 1993; Vayssiére et al., 1994; Zamzami et al., 1995*a*,*b*; Petit et al., 1995; Liu et al., 1996; Schinder et al., 1996; Susin et al., 1996; White and Reynolds, 1996; Ellerby et al., 1997). These findings have led to a focus on potential contributions of mitochondrial dysfunction to neurodegenerative diseases (see Bowling and Beal, 1995). Mitochondria are of particular interest in PD, where evidence has suggested that an underlying deficit of complex I enzyme activity in the mitochondrial electron transport chain exists (Parker et al., 1989; Schapira et al., 1990*a*,*b*; Shoffner et al., 1991; Mann et al., 1992; Martin et al., 1996; Sheehan et al., 1997). Whether this plays a causative role in PD has not yet been elucidated, but it suggests that deficiencies in mitochondrial function could be involved in the degeneration of DA neurons.

Several mitochondrial processes can be disrupted by oxidants such as ROS and quinones. One such process is mitochondrial respiration, which is responsible for generating ATP through oxidative phosphorylation. Several enzymes in the electron transport chain have been shown to be inhibited following exposure to ROS or sulfhydryl-modifying agents (Kenney, 1975; Yagi and Hatefi, 1987; Zhang et al., 1990; Benard and Balasubramanian, 1995). Because both ROS and quinones, formed as a result of DA oxidation, are capable of modifying critical sulfhydryl groups on proteins, these electron transport enzymes may be susceptible to damage by DA oxidation products.

Another potential target of DA oxidation products is the mitochondrial permeability transition pore (PTP). The PTP is a calcium-dependent, proteinaceous pore that allows the normally impermeable inner membrane of mitochondria to become permeable to solutes of <1,500 Da. The change in membrane permeability leads to depolarization of the transmembrane potential, release of small solutes and then proteins, osmotic swelling, and a loss of oxidative phosphorylation (for review, see Gunter and Pfeiffer, 1990). Opening of the PTP has been implicated in several forms of neuronal death including apoptosis, excitotoxicity, ischemia, and toxicity due to the parkinsonian neurotoxin MPTP (Uchino et al., 1995; Nieminen et al., 1996; Packer et al., 1996; Schinder et al., 1996; White and Reynolds, 1996; Zamzami et al., 1996; Ouyang et al., 1997; Cassarino et al., 1998). Many oxidants and toxic quinones are known inducers of PTP opening (e.g., see Gunter and Pfeiffer, 1990). Likewise, sulfhydryl modification has been shown to induce PTP opening, and critical cysteinyl residues have been implicated in regulation of the PTP (Bernardi et al., 1992; Valle et al., 1993; Petronilli et al., 1994). Therefore, the PTP is also a potential target of ROS and quinones formed through both DA oxidation pathways.

In this study, we examined the effects of DA oxidation products on both mitochondrial respiration and the PTP, using isolated respiring brain mitochondria. We report that MAO-catalyzed oxidation of DA and production of H_2O_2 inhibit active mitochondrial respiration, whereas DA quinone production leads to a large increase in resting respiration, indicative of an increase in inner membrane permeability. In addition, we found that the oxidation of DA to DA quinone results in a cyclosporin A (CsA)-inhibitable increase in mitochondrial swelling, suggestive of the opening of the PTP. These effects on mitochondrial function could contribute to DA-induced toxicity and to the neurodegenerative process in PD.

MATERIALS AND METHODS

Mitochondrial isolation

Brain mitochondria were isolated from male Sprague–Dawley rats (300–350 g) by the method of Rosenthal et al. (1987). This method uses 0.02% digitonin to free mitochondria from the synaptosomal fraction. In brief, one rat was decapitated, and the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenized in a Dounce glass tissue homogenizer (via six strokes each with a loose-fitting pestle and then a tight-fitting pestle) at 4°C in 10 ml of isolation medium (225 m*M* mannitol, 75 m*M* sucrose, 5 m*M* HEPES, 1 m*M* EGTA, 1 mg/ml bovine serum albumin, pH 7.4) containing 5 mg of the bacterial protease Nagarse. Single brain homogenates were brought to 30 ml, divided equally into three tubes, and then centrifuged at 2,000 *g* for 3 min. Pellets were resuspended to 10 ml and recentrifuged as above, and the supernatants were pooled and centrifuged in four tubes at 12,000 *g* for 8 min. The pellets, including the fluffy synaptosomal layer, were resuspended in two tubes to 10 ml each in isolation medium containing 0.02% digitonin and centrifuged at 12,000 *g* for 10 min. The brown mitochondrial pellets without the synaptosomal layer were then resuspended again in 10 ml of medium and recentrifuged at 12,000 *g* for 10 min. The mitochondrial pellets were resuspended in 50 μl of medium/tube and combined. Mitochondrial protein yields, determined by the method of Bradford (1976), were ~8–12 mg per rat brain. When utilized, liver mitochondria were isolated from 1.5–1.75 g of liver tissue using the identical procedure, which produced 20–25 mg of mitochondrial protein.

Mitochondrial respiration

Respiration measurements were determined polarographically with a thermostatically controlled (37°C) Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) according to the method of Rosenthal et al. (1987) in standard respiration medium containing 125 m*M* KCl, 2 m*M* K_2HPO_4, 1 m*M* $MgCl_2$, 5 m*M* K-HEPES (pH 7.0), 1 m*M* EGTA, 5 m*M* glutamate, and 5 m*M* malate. Mitochondria (0.5 mg of protein/ml) were added to 1.6 ml of medium in a water-jacketed chamber (Gilson, Middleton, WI, U.S.A.). Ac-

tive state 3 respiration rates were determined by the addition of ADP (0.25 m*M*), and resting state 4 respiration rates were determined after consumption of ADP and the addition of oligomycin (2 μg/ml) to inhibit ATP synthase. Rates are expressed as nanograms of oxygen atoms consumed per minute per milligram of protein and were calculated based on the solubility of oxygen in the air-saturated, temperature-equilibrated medium of 390 ng of O/ml at 37°C and 760 mm Hg. Evaluation of state 3 and state 4 rates occurred over ~3–5 min for each sample. Prior to the initiation of every experiment, respiration rates of the mitochondrial preparation were determined, and mitochondria were used for these studies when the ratio of state 3 respiration to state 4 respiration was determined to be at least 7.0, signifying healthy, well-coupled mitochondria.

For experiments examining the effects of DA oxidation products on mitochondrial respiration, mitochondria (0.5 mg of protein/ml) were incubated in medium alone or medium containing the indicated compounds for 5 min in the electrode chamber at 37°C, with air bubbled into the chamber to maintain O_2 saturation. All control incubations were performed in an identical manner. At the end of the incubation period, state 3 and state 4 respiration was measured as described above. In experiments examining succinate-linked respiration, the medium contained 125 m*M* KCl, 2 m*M* K_2HPO_4, 1 m*M* $MgCl_2$, 5 m*M* K-HEPES (pH 7.0), 1 m*M* EGTA, 5 m*M* succinate, and 2 μ*M* rotenone. For experiments using ascorbate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) to examine complex IV activity, mitochondria were incubated for 5 min in the standard respiration medium containing malate (5 m*M*) and glutamate (5 m*M*). After the incubation period, rotenone (2 μ*M*) was added, followed by the addition of antimycin A (1 μ*M*), ascorbate (2 m*M*), and TMPD (0.1 m*M*), and state 3 respiration was measured with the addition of ADP (0.25 m*M*).

Mitochondrial swelling

Mitochondrial swelling was measured spectrophotometrically (Beckman DU-640, Fullerton, CA, U.S.A.) by monitoring the decrease in absorbance at 540 nm over 10 min similar to previously described methods (Broekemeier et al., 1989). Mitochondria (1 mg of protein) were incubated in 2 ml of medium containing 213 m*M* mannitol, 70 m*M* sucrose, 3 m*M* HEPES (pH. 7.4), 10 m*M* succinate, and 1 μ*M* rotenone. $CaCl_2$ (70 μ*M*) was added after 30 s, and other indicated compounds were added at 2 min. When CsA or GSH was used, it was added to the buffer prior to the addition of the mitochondria. When tyrosinase was used to oxidize DA and when 6-hydroxydopamine (6-OHDA) was used, interfering absorbance due to colored oxidative products was subtracted from measurements using blanks containing only buffer with DA and tyrosinase or buffer with 6-OHDA. Data were quantified and compared by calculating the total decrease in absorbance from 2 to 10 min.

Statistical analysis

Analyses were performed by one-way ANOVA followed by Tukey's post hoc comparisons. A probability of $p < 0.05$ was considered significant. The n values reported refer to data obtained from n separate experiments.

RESULTS

Mitochondrial respiration

A typical measure of oxygen consumption in our isolated brain mitochondrial preparation is shown in Fig. 1. Mitochondrial respiration is conventionally classified into several states, which can be measured via oxygen consumption (Chance and Williams, 1956). State 3, termed active respiration, is defined as respiration in the presence of an oxidizable substrate and ADP and thus is a measure of the respiration that is coupled to ATP synthesis. State 4, or resting, respiration is the rate of respiration in the presence of substrate, but without ADP, and thus is a measure of the rate of respiration that is not coupled to ATP synthesis. Mean rates of active ADP-linked state 3 respiration and resting state 4 respiration were 216 ± 17 and 23 ± 2 ng of O/min/mg of protein, respectively, in untreated brain mitochondria (n = 28; mean ± SEM). The ratio of state 3 to state 4 can be used to evaluate the functional health of the preparation by giving an indication of the degree to which respiration is coupled to ATP synthesis. The average ratio of state 3 to state 4 was 10.2. For experiments in which liver mitochondria were used, mean state 3 and state 4 respiration was 219 ± 44 and 21 ± 5.6 ng of O/min/mg of protein, respectively, and did not differ significantly from that in isolated brain mitochondria (n = 6; mean ± SEM).

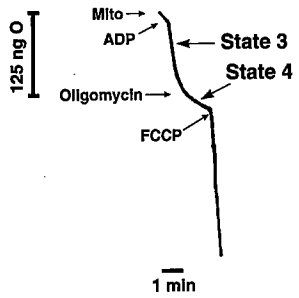

FIG. 1. Representative measure of baseline mitochondrial respiration. For each isolated mitochondrial preparation, oxygen consumption was first measured in isolated brain mitochondria (0.5 mg of protein/ml) with glutamate and malate as substrates, prior to any experimentation, as described in Materials and Methods. State 3 respiration was measured after addition of ADP (0.25 m*M*), and state 4 respiration was measured after addition of oligomycin (2 μg/ml). The rate of uncoupled respiration was recorded after the addition of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 150 n*M*). Mean rates of oxygen consumption were 216 ± 17 and 23 ± 2 ng of O/min/mg of protein for state 3 and state 4, respectively.

Effects of DA oxidation on mitochondrial respiration

We first examined the effect of DA alone on mitochondrial respiration. When mitochondria were incubated for 5 min in the control respiration buffer, mean state 3 respiration was 86 ± 5 ng of O/min/mg of protein, and mean state 4 respiration was 18 ± 3 ng of O/min/mg of protein (Fig. 2). The reduction in state 3 respiration is typical for isolated brain mitochondria after incubation periods at 37°C. When brain mitochondria were incubated for 5 min in respiration buffer containing DA (100 μ*M*), state 3 respiration was reduced by 24% as compared with respiration after incubation in buffer alone (Fig. 2). However, state 4 respiration was unaffected by the presence of DA (Fig. 2). To determine whether the

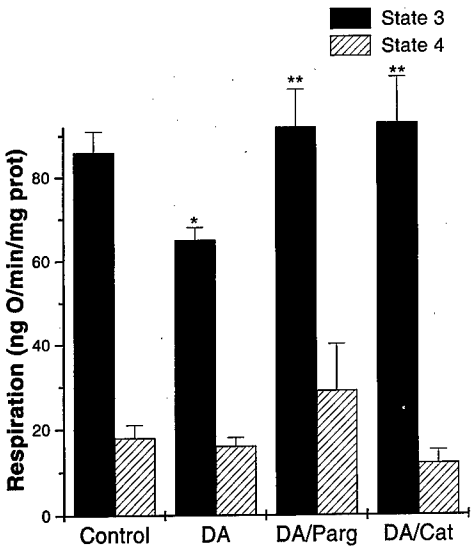

FIG. 2. Effect of DA on mitochondrial respiration. Isolated brain mitochondria were incubated for 5 min as described in Materials and Methods in buffer alone or buffer containing DA (100 μM), DA + pargyline (Parg; 10 μM), or DA + catalase (Cat; 1 U/ml), and then oxygen consumption was measured, utilizing glutamate and malate as substrates. Under control conditions, mean state 3 respiration was 86 ± 5 ng of O/min/mg of protein, and mean state 4 respiration was 18 ± 3 ng of O/min/mg of protein. *Significantly less than respiration under control conditions; **significantly greater than respiration after exposure to DA alone (mean ± SEM; n = 5–16).

state 3 inhibition caused by DA was due to H_2O_2 formed during MAO-catalyzed oxidation of DA, we examined the effects of adding the nonselective MAO inhibitor pargyline (10 μM) or the H_2O_2-scavenging enzyme catalase (1 U/ml) to the incubation medium. We found that the addition of either pargyline or catalase during the incubation with DA completely prevented the inhibition of state 3 respiration (Fig. 2). Neither pargyline nor catalase alone had any effect on mitochondrial respiration (data not shown).

To begin to determine which enzyme complexes of the electron transport chain were affected by DA, we utilized various substrates that donate electrons at different points in the electron transport chain, thereby hoping to bypass the DA-induced inhibition. We observed that when succinate and rotenone were utilized to bypass complex I and donate electrons via complex II to complex III, the decrease in state 3 respiration following incubation with DA was still observed (Fig. 3). Under these conditions, state 3 respiration was inhibited by 37% in the presence of DA as compared with control, an inhibition that was completely prevented by pargyline. However, when ascorbate and TMPD were utilized to donate electrons directly to cytochrome *c*, bypassing complexes I and III, incubation with DA no longer had any effect on state 3 respiration as compared with incubation under the same conditions without DA (Fig. 3).

To examine whether the products resulting from oxidation of the catechol ring would alter mitochondrial respiration, we utilized the enzyme tyrosinase to directly oxidize DA to DA quinone. Mitochondria were incubated for 5 min in standard respiration medium or medium containing DA (20 or 100 μM) and a concentration of tyrosinase (200 mU/ml) that was determined to oxidize all of the DA within 2 min (Berman and Hastings, 1997). Tyrosinase alone had no effect on respiration (data not shown). Following the incubation, we found that incubation with either concentration of DA quinone had no significant effect on state 3 respiration (Fig. 4A). However, we observed a dramatic increase in state 4 respiration with both the lower and the higher DA concentrations (+202 and +280%, respectively; Fig. 4B). Coincubation with GSH (500 μM), which can either reduce the DA quinone to DA or act as a nucleophile to bind DA, prevented the DA oxidation-induced increase in state 4 respiration at both concentrations of DA (Fig. 4B). GSH at this concentration had no effect on respiration (data not shown). When superoxide dismutase (SOD; 1 U/ml) and catalase (1 U/ml) were present, they did not prevent the increase in state 4 respiration caused by DA and tyrosinase (Fig. 4B).

Effects of DA oxidation products on mitochondrial permeability transition

Induction of permeability transition has been shown to lead to swelling of mitochondria (Gunter and Pfeiffer, 1990), which can be measured spectrophotometrically. Figure 5A shows the results of a representative swelling experiment. Exposure of brain mitochondria to control

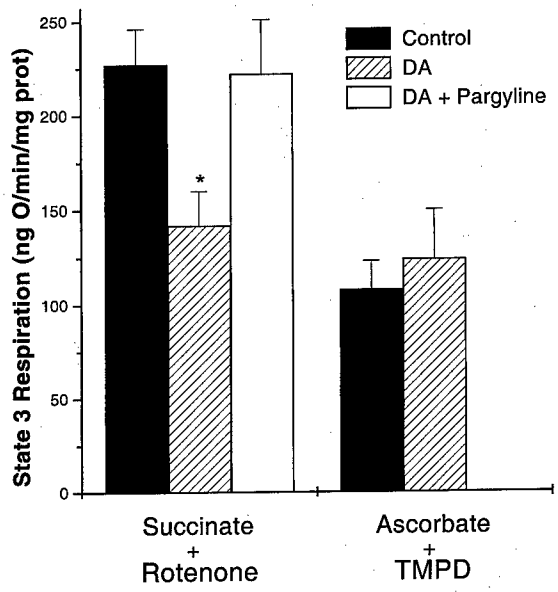

FIG. 3. Effect of bypassing steps in the electron transport chain on DA-induced inhibition of state 3 respiration. Mitochondria were incubated in buffer alone or buffer containing DA (100 μM) or DA + pargyline (10 μM) as described in Materials and Methods. Succinate (5 mM) + rotenone (2 μM) were utilized to donate electrons to complex II, and ascorbate (2 mM) + TMPD (100 μM) + antimycin A (1 μM) were utilized to donate electrons to cytochrome *c*. *Significantly less than respiration under control conditions (mean ± SEM; n = 3–8).

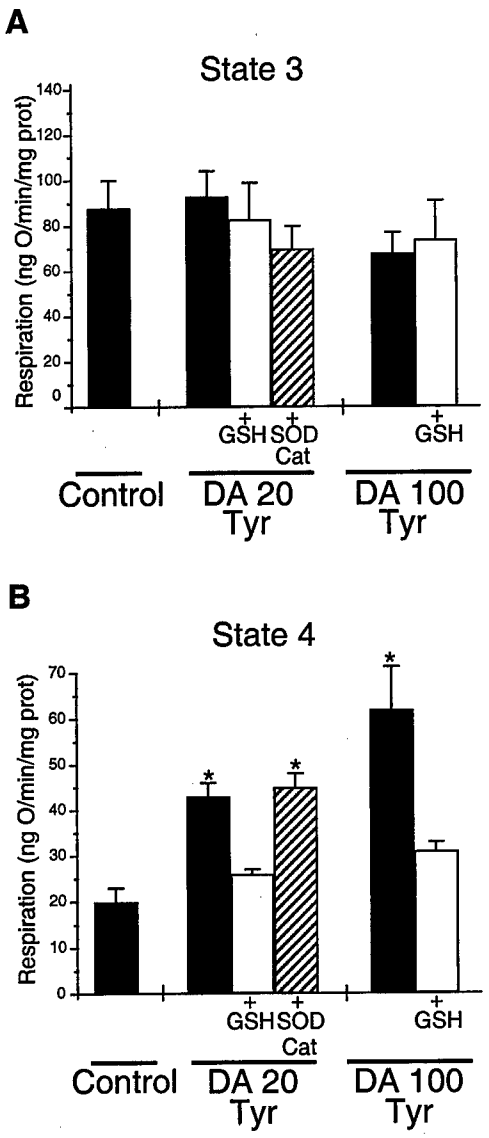

FIG. 4. Effect of enzymatic oxidation of DA on mitochondrial respiration. Mitochondria were incubated for 5 min in buffer or buffer containing DA (20 or 100 μM) + tyrosinase (Tyr; 200 U/ml) with or without GSH (500 μM) or SOD (1 U/ml) + catalase (Cat; 1 U/ml), and then respiration was measured using glutamate and malate as substrates. **A:** State 3 respiration. **B:** State 4 respiration. *Significantly different from control respiration (mean ± SEM; n = 3–13).

conditions (70 μM $CaCl_2$) led to a small degree of mitochondrial swelling. Exposure to 70 μM $CaCl_2$, followed by the addition of DA (100 μM), did not lead to an increase in the amount of mitochondrial swelling. However, exposure to the rapidly oxidizing catecholamine 6-OHDA (100 μM) significantly increased mitochondrial swelling. The results of these experiments, expressed as a change in absorbance, are quantified in Fig. 5B, demonstrating that 6-OHDA caused a threefold increase in mitochondrial swelling above control levels. CsA (850 nM), known to prevent the opening of the PTP in liver and heart mitochondria (Fournier et al., 1987; Crompton et al., 1988; Broekemeier et al., 1989), did not significantly prevent the swelling caused by 6-OHDA (Fig. 5B). CsA alone has no effect on swelling (data not shown).

We also examined the effects of DA quinone production using tyrosinase (Fig. 6). We found that brain mitochondria exposed to $CaCl_2$ (70 μM) followed by DA (100 μM) and tyrosinase (200 U/ml) led to a significant increase in mitochondrial swelling. In contrast to the findings with 6-OHDA, CsA (850 nM) was able to completely prevent the increase in swelling. Similar results were observed when a lower concentration of DA (20 μM) was utilized (Fig. 6). The addition of GSH (1

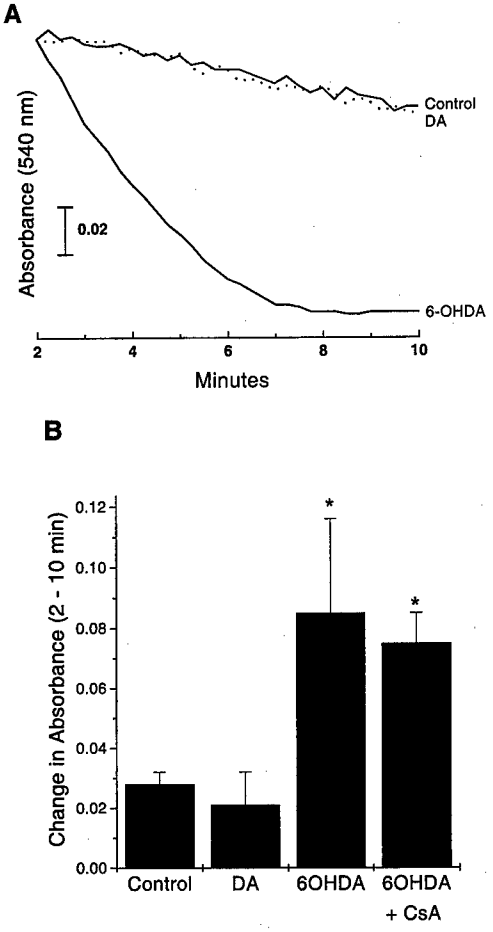

FIG. 5. Effect of DA and 6-OHDA on swelling of brain mitochondria. **A:** Representative traces of the change in absorbance at 540 nm, indicative of swelling in brain mitochondria, are shown following exposure to $CaCl_2$ (70 μM) alone (Control), $CaCl_2$ + DA (100 μM), or $CaCl_2$ + 6-OHDA (100 μM). $CaCl_2$ was added after 0.5 min, and DA or 6-OHDA was added at 2 min. When utilized, CsA (850 nM) was present at the beginning of the experiment. Absorbance changes due to the autoxidation of 6-OHDA were subtracted using blanks containing only buffer and 6-OHDA. **B:** Quantification of swelling measured as the absolute change in absorbance from the time the inducer was added (2 min) to 10 min (mean ± SEM; n = 3–6). *Significantly different from control values ($p < 0.05$).

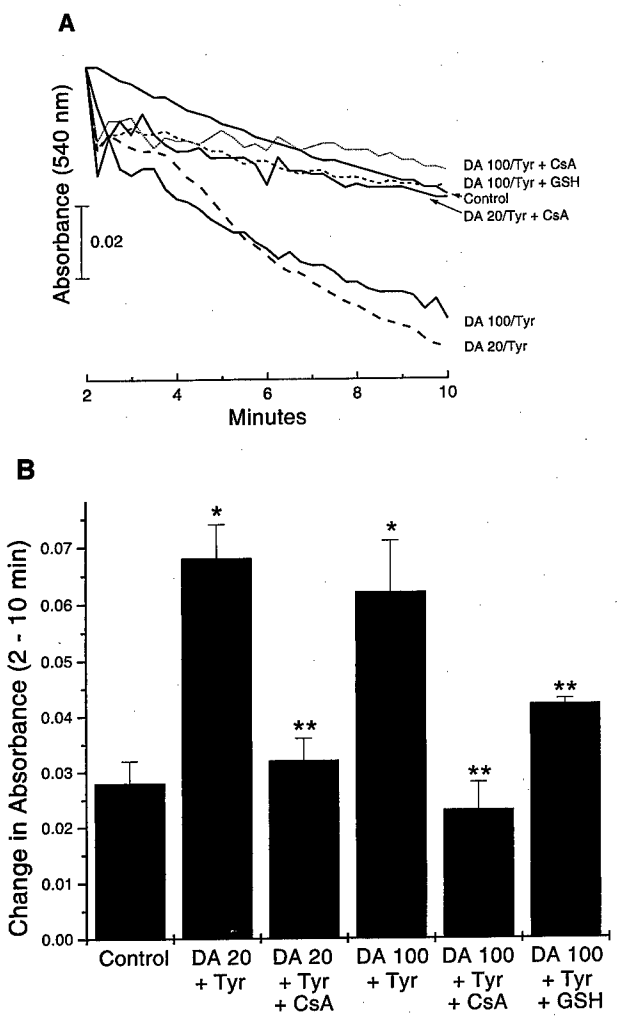

FIG. 6. Effect of tyrosinase (Tyr)-catalyzed oxidation of DA on swelling of brain mitochondria. **A:** Representative traces of swelling after exposure to $CaCl_2$ (70 μM) alone (Control) or + DA and tyrosinase (200 U/ml), with or without the addition of GSH (1 m*M*) or CsA (850 n*M*). $CaCl_2$ was added after 30 s, and then DA and tyrosinase were added at 2 min. When utilized, CsA and GSH were present at the beginning of the experiment. **B:** Quantification of swelling, measured as described in Fig. 5 (mean ± SEM; n = 3–6). *Significantly different from control values; **significantly different from the same condition without CsA or GSH.

m*M*) also significantly reduced the amount of swelling induced by DA and tyrosinase to levels similar to controls (Fig. 6).

The degree of swelling induced by DA oxidation in brain mitochondria is much smaller than that classically observed in studies of inducers of the transition pore. Most compounds have been tested in liver mitochondria, and we have noted that brain mitochondria respond to a much smaller degree to classic inducers of the PTP than do liver mitochondria (S. B. Berman, S. C. Watson, and T. G. Hastings, unpublished data). Therefore, we also examined the effect of DA quinone production on liver mitochondria. We observed that exposure of liver mitochondria to $CaCl_2$ (70 μM) followed by DA (100 μM) and tyrosinase (200 U/ml) resulted in large-amplitude swelling that was also prevented by the addition of CsA (Fig. 7). The change in absorbance induced in liver mitochondria by DA and tyrosinase was 11-fold higher than that observed in brain mitochondria (Figs. 6B and 7B) and was of similar magnitude to that observed for classic inducers of the PTP in liver mitochondria (e.g., Savage et al., 1991; Bernardi et al., 1992; S. B. Berman, S. C. Watson, and T. G. Hastings, unpublished data).

DISCUSSION

In this study, we provide evidence that both oxidation of the DA catechol ring and MAO-catalyzed DA oxidation can negatively affect the function of isolated brain mitochondria. We found that exposure of brain mitochondria to DA oxidation products altered both state 3 and state 4 mitochondrial respiration and caused mito-

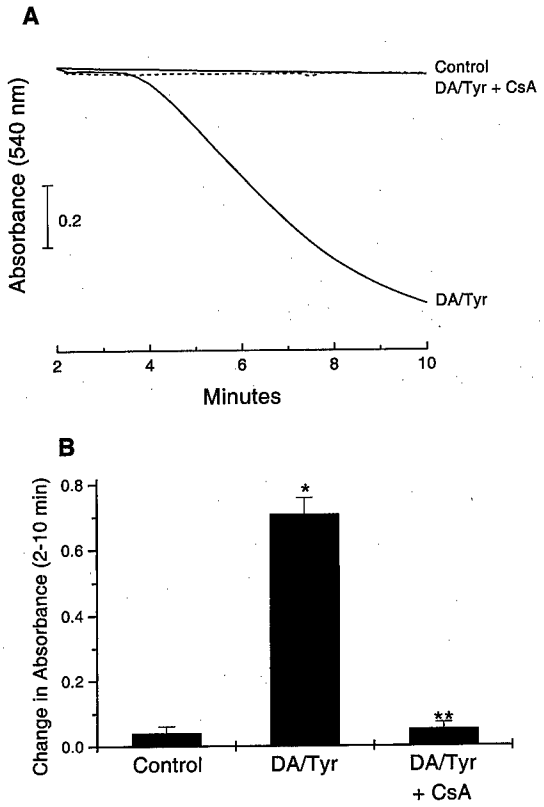

FIG. 7. Effect of tyrosinase (Tyr)-catalyzed oxidation of DA on swelling of liver mitochondria. **A:** Representative traces of swelling after exposure to $CaCl_2$ (70 μM) alone (Control) or + DA and tyrosinase (200 U/ml), with or without CsA (850 n*M*). Experiments were conducted as described in Fig. 6, except utilizing mitochondria isolated from liver. **B:** Quantification of swelling, measured as described in Fig. 5 (mean ± SEM; n = 3–6). *Significantly different from control values ($p < 0.05$); **significantly different from the same condition without CsA ($p < 0.05$). Note the different scale indicating absorbance change as compared with Fig. 6.

chondrial swelling that may be indicative of the opening of the PTP.

Mitochondrial respiration

DA oxidation to DA quinone. We provide the first evidence that oxidation of DA to DA quinone can lead to changes in mitochondrial respiration. We found that exposure of mitochondria to DA quinone resulted in a large increase in the resting rate of mitochondrial respiration (state 4). Normally, the transport of electrons through the electron transport chain is coupled to the pumping of protons from the matrix across the inner mitochondrial membrane, which is generally impermeable to protons (Brand et al., 1994). This establishes a proton gradient across the membrane, and protons are driven back into the matrix through the ATP synthase, providing the energy to convert ADP to ATP. In this way, mitochondrial respiration and its resultant oxygen consumption are coupled to ATP synthesis. If mitochondrial respiration were completely coupled to ATP synthesis, resting state 4 respiration would be zero, as ATP synthesis is not occurring. In healthy mitochondria, a low rate of respiration persists under state 4 conditions, and this is thought to be indicative of a low rate of proton leakage back across the inner membrane (Hafner et al., 1990). An increase in state 4 respiration therefore implies an increase in the proton leak across the membrane. After exposure to DA quinone, the rate of state 4 respiration increases nearly to the level of respiration measured in the presence of ADP (state 3). In other words, after exposure to DA quinone, respiration in the mitochondria proceeded without being coupled to ATP synthesis, an unproductive use of cellular energy.

It is possible that tyrosinase-catalyzed oxidation of DA produces not only the DA quinone but also $O_2^{\bullet -}$, albeit at a much lower level (Tomita et al., 1984; however, see Koga et al., 1992). To begin to determine whether the quinone is important in the effect on mitochondrial respiration that was observed, we investigated protection by either GSH or SOD plus catalase. GSH can reduce the quinone to DA or utilize its sulfhydryl group to covalently bind to DA quinone (Tse et al., 1976), thus scavenging the quinone and preventing it from binding to mitochondrial proteins. GSH was largely able to prevent the effect of DA and tyrosinase, suggesting that DA quinone is involved in the increase in resting respiration. SOD and catalase, which would detoxify any $O_2^{\bullet -}$ that might be formed, showed no protection against the effect caused by DA plus tyrosinase, further supporting the hypothesis that it is the DA quinone that is responsible for the uncoupling of respiration from ATP production.

Although the mechanism associated with the stimulation of resting respiration by DA quinone is not known, a similar effect on brain mitochondria was recently reported after exposure to the oxidant peroxynitrite (Brookes et al., 1998). This effect was prevented in the presence of the antioxidant Trolox. Although lipid peroxidation was suggested as a possible cause of the peroxynitrite effect, peroxynitrite, like DA quinone, can also modify sulfhydryl groups (Radi et al., 1991). Thus, it is also possible that covalent modification of sulfhydryl-containing proteins in the mitochondrial membrane contributes to the increase in proton permeability.

MAO-catalyzed oxidation of DA. We also found that MAO-catalyzed oxidation of DA, producing H_2O_2, led to an inhibition of active state 3 mitochondrial respiration. As both catalase and pargyline could prevent the inhibition, the production of H_2O_2 appears to be responsible for the effect. To determine where in the electron transport chain the inhibition was occurring, we utilized compounds that donate electrons directly to specific points along the chain, bypassing others. We found that inhibition of state 3 persisted when complex I was bypassed, suggesting that a process downstream of complex I is being affected. Furthermore, the inhibition disappeared when electrons were directly donated to cytochrome *c* just prior to complex IV, thus bypassing complex I and III. These findings suggest the possibility that the ubiquinone–complex III component of the electron transport chain is the target of the H_2O_2-induced inhibition. However, we cannot exclude the possibility that both complex I and II are inhibited by the H_2O_2. Both succinate dehydrogenase (complex II) and NADH dehydrogenase (complex I) have been shown to have thiol-dependent activity (Kenney, 1975; Benard and Balasubramanian, 1995) and thus may be susceptible to oxidation by H_2O_2.

This is the first study to examine the effect of DA on the oxygen consumption of well-coupled, healthy, intact mitochondria. Several previous studies examined only the effect of DA on complex I enzyme activity in tissue homogenates and reported conflicting results such as complete inhibition of complex I by 10 μM DA (Ben-Shachar et al., 1995), 25–50% inhibition with 1–100 mM DA (Przedborski et al., 1993), and 10% inhibition by 10 mM DA (Morikawa et al., 1996). The reasons for the discrepancies are not clear but may reflect different assay conditions. The two studies that began to investigate the role of DA oxidation in the enzyme inhibition noted prevention by antioxidants (Przedborski et al., 1993) and iron chelation (Ben-Shachar et al., 1995) but not by MAO inhibition (Ben-Shachar et al., 1995). All of these studies utilized only disrupted mitochondrial preparations to examine enzyme activities. However, studies utilizing intact, actively respiring mitochondria are likely to be more closely related to physiological circumstances, as the electron transport chain enzymes are normally coupled to ATP synthesis. In fact, studies have suggested that in nonsynaptic brain mitochondria, at least 70% inhibition of complex I is required before changes in state 3 respiration or ATP production are observed (Davey and Clark, 1996). Thus, the small inhibitory effect on respiration observed in our study may be indicative of larger changes in enzyme function. One other recent study utilized intact mitochondria but used extended incubation periods, and therefore less-coupled mitochondria, and utilized dye reduction rather than oxygen consumption as a measure of respiration, which does not allow examination of ATP synthesis-coupled

respiration (Cohen et al., 1997). It also studied only one of the two physiologic pathways of DA oxidation, MAO-catalyzed oxidation of DA, and reported an inhibition of respiration dependent on this oxidation, similar to our results. With avoidance of some of these limitations in our methods, the results of the current study confirm this finding as well as demonstrate significant effects on respiration by DA oxidation to DA quinone.

Mitochondrial permeability transition

Opening of the mitochondrial PTP has been suggested to play a critical role in several forms of neuronal cell death, including excitotoxicity, ischemia, MPP^+-induced toxicity, and hypoglycemia (Uchino et al., 1995; Nieminen et al., 1996; Packer et al., 1996; Schinder et al., 1996; White and Reynolds, 1996; Ouyang et al., 1997; Friberg et al., 1998). The increase in inner mitochondrial membrane permeability that is induced by PTP opening leads to mitochondrial membrane depolarization, release of small solutes and proteins, osmotic swelling, and a loss of oxidative phosphorylation (see Gunter and Pfeiffer, 1990; Bernardi, 1995). The PTP has been shown to contain critical thiol residues (Chernyak and Bernardi, 1996), and it has been induced by many oxidants including quinones (Gunter and Pfeiffer, 1990; Henry and Wallace, 1995; Henry et al., 1995). Therefore, we also examined effects of DA oxidation products on the PTP in mitochondria, and we conclude that DA quinone production resulted in the opening of the mitochondrial PTP. Mitochondrial swelling was induced in brain mitochondria after exposure to DA quinone that was completely prevented by the presence of the PTP inhibitor CsA, suggesting involvement of the PTP. GSH, which can inhibit DA quinone from reacting with sulfhydryl-containing mitochondrial proteins by covalently binding it as well as reducing it, was also able to prevent the swelling induced by DA quinone production.

Classically, most studies of the pore are performed in liver mitochondria, where a robust response has been well characterized (see Bernardi, 1995). To further investigate the effect of DA quinone production on classic pore characteristics, we also examined liver mitochondria. We found that enzymatic oxidation of DA produced large-amplitude swelling of liver mitochondria that was completely prevented by CsA, similar to that observed with other known pore inducers (e.g., Broekemeier et al., 1989; Bernardi et al., 1992). The magnitude of the swelling response in brain mitochondria was much smaller than that observed in liver (~10% of liver response) but is consistent with comparisons of PTP opening in brain and liver mitochondria after exposure to classic pore inducers such as calcium with phosphate (S. B. Berman, S. C. Watson, and T. G. Hastings, unpublished data).

The opening of the PTP induced by DA oxidation to DA quinone is interesting, given recent mechanistic studies of the transition pore. Evidence suggests that two sites exist on pore proteins that are important regulators of PTP function (Chernyak and Bernardi, 1996). One site contains vicinal thiols, which, when oxidized to disulfides, induce PTP opening. This site can be protected from oxidation by compounds that bind monothiols and prevent disulfide formation (Petronilli et al., 1994; Chernyak and Bernardi, 1996). One might expect that binding of DA quinone could protect similarly, binding directly to the monothiols. Benzoquinone has been suggested to inhibit PTP opening through this mechanism (Palmeira and Wallace, 1997). In contrast, there is also evidence that high concentrations of a monothiol-binding compound, *N*-ethylmaleimide, increased rather than decreased PTP opening (Petronilli et al., 1994), similar to the results with DA quinone.

A second important regulatory modulation involves the redox status of pyridine nucleotides. Oxidation of NADH and NADPH also increases pore opening, through an unknown mechanism that has been shown to be independent of the dithiol site (Chernyak and Bernardi, 1996). Oxidation of pyridines (both NADH and NADPH) can occur enzymatically through the cytosolic and mitochondrial enzyme DT-diaphorase, which reduces quinones via a two-electron reduction (Cadenas, 1995), and it has been shown that quinone substrates of DT-diaphorase can induce PTP opening (Chernyak and Bernardi, 1996). PTP opening by DA quinone could also be explained by this enzyme converting DA quinone to DA, oxidizing pyridine nucleotides in the process and increasing the probability of pore opening.

Availability of DA

For DA oxidation products to exert effects on mitochondrial function, DA must be available to mitochondrial proteins. Although the majority of DA in DA neurons is stored in vesicles, much of the DA clearly has access to mitochondria, because MAO, the major metabolizing enzyme of DA, is located on the outer mitochondrial membrane (Greenawalt and Schnaitman, 1970). One could hypothesize that under conditions of increased availability of cytoplasmic DA or increased synthesis and metabolism of DA, the potential for DA oxidation-induced effects on mitochondria would increase. Such conditions are thought to exist both in PD, where there is an increase in DA turnover (Bernheimer et al., 1973), and following high doses of methamphetamine, resulting in the redistribution of DA from vesicular storage to the cytoplasm (Cubbells et al., 1994; Sulzer et al., 1995). In fact, DA oxidation products have been shown to be increased in the substantia nigra of postmortem brain tissue from parkinsonian patients (Fornstedt et al., 1989; Spencer et al., 1998) and in rat striatum following exposure to methamphetamine (LaVoie and Hastings, 1999). Thus, these conditions may lead to an increase in cytoplasmic DA and subsequent increase in DA oxidation products, resulting in eventual dysfunction of mitochondria. Although this study was performed in vitro with isolated mitochondria and relatively high concentrations of DA, it raises the possibility that DA oxidation-induced alterations in mitochondrial function could occur under pathological conditions.

Conclusions

The alterations in mitochondrial function due to DA oxidation have several potential implications for neuronal cell death and neurodegenerative disease. As mentioned previously, it has been reported that individuals with PD exhibit a deficiency in the activity of complex I of the electron transport chain. It is not entirely clear whether this is a deficiency in all cells (Parker et al., 1989; Shoffner et al., 1991; Martin et al., 1996; Sheehan et al., 1997) or is limited to the substantia nigra (Schapira et al., 1990*b*; Mann et al., 1992), but the majority of evidence seems to point to a global deficiency. Therefore, the question arises as to the mechanism by which an underlying enzyme deficiency in all cells would lead to the specific loss of DA neurons. Our evidence suggests that one potential contributing factor may be the presence of DA. It is possible that an underlying deficiency, which alone does not cause cell death, is exacerbated by the presence of reactive DA metabolites. Other factors that have been implicated in PD, such as decreased antioxidant ability or increased iron (see Fahn and Cohen, 1992), also will contribute to increases in DA oxidation.

The effects of DA oxidation on mitochondrial function may also contribute to more acute neurotoxic events in which DA has been implicated. In methamphetamine toxicity, for example, DA is known to be important to the toxicity (Cubbels et al., 1994; Stephans and Yamamoto, 1994), and DA oxidation products have been shown to correlate with methamphetamine toxicity (LaVoie and Hastings, 1999). Thus, DA-induced mitochondrial dysfunction may also play a role in this neurotoxicity.

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Dopamine Oxidation Alters Mitochondrial Respiration and Induces Permeability Transition in Brain Mitochondria: Implications for Parkinson's Disease

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Abstract: Both reactive dopamine metabolites and mitochondrial dysfunction have been implicated in the neurodegeneration of Parkinson's disease. Dopamine metabolites, dopamine quinone and reactive oxygen species, can directly alter protein function by oxidative modifications, and several mitochondrial proteins may be targets of this oxidative damage. In this study, we examined, using isolated brain mitochondria, whether dopamine oxidation products alter mitochondrial function. We found that exposure to dopamine quinone caused a large increase in mitochondrial resting state 4 respiration. This effect was prevented by GSH but not superoxide dismutase and catalase. In contrast, exposure to dopamine and monoamine oxidase-generated hydrogen peroxide resulted in a decrease in active state 3 respiration. This inhibition was prevented by both pargyline and catalase. We also examined the effects of dopamine oxidation products on the opening of the mitochondrial permeability transition pore, which has been implicated in neuronal cell death. Dopamine oxidation to dopamine quinone caused a significant increase in swelling of brain and liver mitochondria. This was inhibited by both the pore inhibitor cyclosporin A and GSH, suggesting that swelling was due to pore opening and related to dopamine quinone formation. In contrast, dopamine and endogenous monoamine oxidase had no effect on mitochondrial swelling. These findings suggest that mitochondrial dysfunction induced by products of dopamine oxidation may be involved in neurodegenerative conditions such as Parkinson's disease and methamphetamine-induced neurotoxicity. **Key Words:** Dopamine—Mitochondria—Permeability transition—Parkinson's disease—Quinone—Respiration.
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In Parkinson's disease (PD), the cause of the degeneration of dopaminergic neurons of the substantia nigra is unknown, but evidence suggests that oxidative stress is involved (for review, see Fahn and Cohen, 1992). One source of oxidative stress that is unique to dopaminergic neurons is the presence of dopamine (DA) itself, as DA can form reactive oxygen species (ROS) and quinones through two different pathways. First, DA is metabolized via monoamine oxidase (MAO) to produce hydrogen peroxide (H_2O_2) and dihydroxyphenylacetic acid (Maker et al., 1981). H_2O_2, if not reduced by cellular antioxidant mechanisms such as GSH and GSH peroxidase, can react with transition metals such as iron to form hydroxyl radical (Halliwell, 1992). This molecule will immediately react with lipids, DNA, and susceptible amino acids in proteins, thus causing cellular damage (Halliwell, 1992). Second, the catechol ring of DA can undergo oxidation to form DA quinone and ROS such as H_2O_2 and superoxide anion ($O_2^{\bullet -}$) in a reaction that can occur either spontaneously in the presence of transition metals or enzymatically (Graham, 1978; Hastings, 1995). The DA quinone is electron-deficient and reacts readily with cellular nucleophiles such as sulfhydryl groups on free cysteine, GSH, and cysteinyl residues in proteins (Tse et al., 1976; Graham, 1978). The reaction between the DA quinone and sulfhydryl groups leads to covalent modification of protein and free thiols, forming cysteinyl–DA conjugates (Tse et al., 1976; Graham, 1978; Fornstedt et al., 1990; Hastings and Zigmond, 1994). Because free thiols are important antioxidants in cells and protein cysteinyl residues often play critical roles in protein function, alterations of either free or protein thiols could lead to cellular toxicity.

DA is known to be toxic both in vitro (Graham, 1978; Michel and Hefti, 1990) and in vivo (Filloux and Townsend, 1993; Hastings et al., 1996), and we have shown that the formation of cysteinyl–DA conjugates

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Abbreviations used: CsA, cyclosporin A; DA, dopamine; H_2O_2, hydrogen peroxide; MAO, monoamine oxidase; $O_2^{\bullet -}$, superoxide anion; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; PTP, permeability transition pore; ROS, reactive oxygen species; SOD, superoxide dismutase; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

correlates with DA-induced neurotoxicity (Hastings et al., 1996). In addition, we have shown that DA oxidation products can inhibit the function of specific proteins, the DA and glutamate transporters (Berman et al., 1996; Berman and Hastings, 1997), and others have recently reported similar effects on the activities of tryptophan hydroxylase (Kuhn and Arthur, 1998) and tyrosine hydroxylase (Xu et al., 1998).

The protein targets that are critical to the toxicity induced by DA are not yet known, but likely candidates include many of the proteins important in mitochondrial processes. The critical role of mitochondria for cellular survival is well known, and mitochondrial dysfunction has recently been elucidated as an essential target in the induction of apoptosis as well as in excitotoxic neuronal death (Deckwerth and Johnson, 1993; Vayssiére et al., 1994; Zamzami et al., 1995*a*,*b*; Petit et al., 1995; Liu et al., 1996; Schinder et al., 1996; Susin et al., 1996; White and Reynolds, 1996; Ellerby et al., 1997). These findings have led to a focus on potential contributions of mitochondrial dysfunction to neurodegenerative diseases (see Bowling and Beal, 1995). Mitochondria are of particular interest in PD, where evidence has suggested that an underlying deficit of complex I enzyme activity in the mitochondrial electron transport chain exists (Parker et al., 1989; Schapira et al., 1990*a*,*b*; Shoffner et al., 1991; Mann et al., 1992; Martin et al., 1996; Sheehan et al., 1997). Whether this plays a causative role in PD has not yet been elucidated, but it suggests that deficiencies in mitochondrial function could be involved in the degeneration of DA neurons.

Several mitochondrial processes can be disrupted by oxidants such as ROS and quinones. One such process is mitochondrial respiration, which is responsible for generating ATP through oxidative phosphorylation. Several enzymes in the electron transport chain have been shown to be inhibited following exposure to ROS or sulfhydryl-modifying agents (Kenney, 1975; Yagi and Hatefi, 1987; Zhang et al., 1990; Benard and Balasubramanian, 1995). Because both ROS and quinones, formed as a result of DA oxidation, are capable of modifying critical sulfhydryl groups on proteins, these electron transport enzymes may be susceptible to damage by DA oxidation products.

Another potential target of DA oxidation products is the mitochondrial permeability transition pore (PTP). The PTP is a calcium-dependent, proteinaceous pore that allows the normally impermeable inner membrane of mitochondria to become permeable to solutes of $<1,500$ Da. The change in membrane permeability leads to depolarization of the transmembrane potential, release of small solutes and then proteins, osmotic swelling, and a loss of oxidative phosphorylation (for review, see Gunter and Pfeiffer, 1990). Opening of the PTP has been implicated in several forms of neuronal death including apoptosis, excitotoxicity, ischemia, and toxicity due to the parkinsonian neurotoxin MPTP (Uchino et al., 1995; Nieminen et al., 1996; Packer et al., 1996; Schinder et al., 1996; White and Reynolds, 1996; Zamzami et al., 1996; Ouyang et al., 1997; Cassarino et al., 1998). Many oxidants and toxic quinones are known inducers of PTP opening (e.g., see Gunter and Pfeiffer, 1990). Likewise, sulfhydryl modification has been shown to induce PTP opening, and critical cysteinyl residues have been implicated in regulation of the PTP (Bernardi et al., 1992; Valle et al., 1993; Petronilli et al., 1994). Therefore, the PTP is also a potential target of ROS and quinones formed through both DA oxidation pathways.

In this study, we examined the effects of DA oxidation products on both mitochondrial respiration and the PTP, using isolated respiring brain mitochondria. We report that MAO-catalyzed oxidation of DA and production of H_2O_2 inhibit active mitochondrial respiration, whereas DA quinone production leads to a large increase in resting respiration, indicative of an increase in inner membrane permeability. In addition, we found that the oxidation of DA to DA quinone results in a cyclosporin A (CsA)-inhibitable increase in mitochondrial swelling, suggestive of the opening of the PTP. These effects on mitochondrial function could contribute to DA-induced toxicity and to the neurodegenerative process in PD.

MATERIALS AND METHODS

Mitochondrial isolation

Brain mitochondria were isolated from male Sprague–Dawley rats (300–350 g) by the method of Rosenthal et al. (1987). This method uses 0.02% digitonin to free mitochondria from the synaptosomal fraction. In brief, one rat was decapitated, and the whole brain minus the cerebellum was rapidly removed, washed, minced, and homogenized in a Dounce glass tissue homogenizer (via six strokes each with a loose-fitting pestle and then a tight-fitting pestle) at 4°C in 10 ml of isolation medium (225 m*M* mannitol, 75 m*M* sucrose, 5 m*M* HEPES, 1 m*M* EGTA, 1 mg/ml bovine serum albumin, pH 7.4) containing 5 mg of the bacterial protease Nagarse. Single brain homogenates were brought to 30 ml, divided equally into three tubes, and then centrifuged at 2,000 *g* for 3 min. Pellets were resuspended to 10 ml and recentrifuged as above, and the supernatants were pooled and centrifuged in four tubes at 12,000 *g* for 8 min. The pellets, including the fluffy synaptosomal layer, were resuspended in two tubes to 10 ml each in isolation medium containing 0.02% digitonin and centrifuged at 12,000 *g* for 10 min. The brown mitochondrial pellets without the synaptosomal layer were then resuspended again in 10 ml of medium and recentrifuged at 12,000 *g* for 10 min. The mitochondrial pellets were resuspended in 50 μl of medium/tube and combined. Mitochondrial protein yields, determined by the method of Bradford (1976), were ~8–12 mg per rat brain. When utilized, liver mitochondria were isolated from 1.5–1.75 g of liver tissue using the identical procedure, which produced 20–25 mg of mitochondrial protein.

Mitochondrial respiration

Respiration measurements were determined polarographically with a thermostatically controlled (37°C) Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) according to the method of Rosenthal et al. (1987) in standard respiration medium containing 125 m*M* KCl, 2 m*M* K_2HPO_4, 1 m*M* $MgCl_2$, 5 m*M* K-HEPES (pH 7.0), 1 m*M* EGTA, 5 m*M* glutamate, and 5 m*M* malate. Mitochondria (0.5 mg of protein/ml) were added to 1.6 ml of medium in a water-jacketed chamber (Gilson, Middleton, WI, U.S.A.). Ac-

tive state 3 respiration rates were determined by the addition of ADP (0.25 m*M*), and resting state 4 respiration rates were determined after consumption of ADP and the addition of oligomycin (2 μg/ml) to inhibit ATP synthase. Rates are expressed as nanograms of oxygen atoms consumed per minute per milligram of protein and were calculated based on the solubility of oxygen in the air-saturated, temperature-equilibrated medium of 390 ng of O/ml at 37°C and 760 mm Hg. Evaluation of state 3 and state 4 rates occurred over ~3–5 min for each sample. Prior to the initiation of every experiment, respiration rates of the mitochondrial preparation were determined, and mitochondria were used for these studies when the ratio of state 3 respiration to state 4 respiration was determined to be at least 7.0, signifying healthy, well-coupled mitochondria.

For experiments examining the effects of DA oxidation products on mitochondrial respiration, mitochondria (0.5 mg of protein/ml) were incubated in medium alone or medium containing the indicated compounds for 5 min in the electrode chamber at 37°C, with air bubbled into the chamber to maintain O_2 saturation. All control incubations were performed in an identical manner. At the end of the incubation period, state 3 and state 4 respiration was measured as described above. In experiments examining succinate-linked respiration, the medium contained 125 m*M* KCl, 2 m*M* K_2HPO_4, 1 m*M* $MgCl_2$, 5 m*M* K-HEPES (pH 7.0), 1 m*M* EGTA, 5 m*M* succinate, and 2 μ*M* rotenone. For experiments using ascorbate and *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) to examine complex IV activity, mitochondria were incubated for 5 min in the standard respiration medium containing malate (5 m*M*) and glutamate (5 m*M*). After the incubation period, rotenone (2 μ*M*) was added, followed by the addition of antimycin A (1 μ*M*), ascorbate (2 m*M*), and TMPD (0.1 m*M*), and state 3 respiration was measured with the addition of ADP (0.25 m*M*).

Mitochondrial swelling

Mitochondrial swelling was measured spectrophotometrically (Beckman DU-640, Fullerton, CA, U.S.A.) by monitoring the decrease in absorbance at 540 nm over 10 min similar to previously described methods (Broekemeier et al., 1989). Mitochondria (1 mg of protein) were incubated in 2 ml of medium containing 213 m*M* mannitol, 70 m*M* sucrose, 3 m*M* HEPES (pH. 7.4), 10 m*M* succinate, and 1 μ*M* rotenone. $CaCl_2$ (70 μ*M*) was added after 30 s, and other indicated compounds were added at 2 min. When CsA or GSH was used, it was added to the buffer prior to the addition of the mitochondria. When tyrosinase was used to oxidize DA and when 6-hydroxydopamine (6-OHDA) was used, interfering absorbance due to colored oxidative products was subtracted from measurements using blanks containing only buffer with DA and tyrosinase or buffer with 6-OHDA. Data were quantified and compared by calculating the total decrease in absorbance from 2 to 10 min.

Statistical analysis

Analyses were performed by one-way ANOVA followed by Tukey's post hoc comparisons. A probability of $p < 0.05$ was considered significant. The n values reported refer to data obtained from n separate experiments.

RESULTS

Mitochondrial respiration

A typical measure of oxygen consumption in our isolated brain mitochondrial preparation is shown in Fig. 1. Mitochondrial respiration is conventionally classified into several states, which can be measured via oxygen consumption (Chance and Williams, 1956). State 3, termed active respiration, is defined as respiration in the presence of an oxidizable substrate and ADP and thus is a measure of the respiration that is coupled to ATP synthesis. State 4, or resting, respiration is the rate of respiration in the presence of substrate, but without ADP, and thus is a measure of the rate of respiration that is not coupled to ATP synthesis. Mean rates of active ADP-linked state 3 respiration and resting state 4 respiration were 216 ± 17 and 23 ± 2 ng of O/min/mg of protein, respectively, in untreated brain mitochondria (n = 28; mean ± SEM). The ratio of state 3 to state 4 can be used to evaluate the functional health of the preparation by giving an indication of the degree to which respiration is coupled to ATP synthesis. The average ratio of state 3 to state 4 was 10.2. For experiments in which liver mitochondria were used, mean state 3 and state 4 respiration was 219 ± 44 and 21 ± 5.6 ng of O/min/mg of protein, respectively, and did not differ significantly from that in isolated brain mitochondria (n = 6; mean ± SEM).

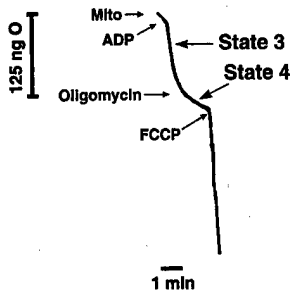

FIG. 1. Representative measure of baseline mitochondrial respiration. For each isolated mitochondrial preparation, oxygen consumption was first measured in isolated brain mitochondria (0.5 mg of protein/ml) with glutamate and malate as substrates, prior to any experimentation, as described in Materials and Methods. State 3 respiration was measured after addition of ADP (0.25 m*M*), and state 4 respiration was measured after addition of oligomycin (2 μg/ml). The rate of uncoupled respiration was recorded after the addition of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 150 n*M*). Mean rates of oxygen consumption were 216 ± 17 and 23 ± 2 ng of O/min/mg of protein for state 3 and state 4, respectively.

Effects of DA oxidation on mitochondrial respiration

We first examined the effect of DA alone on mitochondrial respiration. When mitochondria were incubated for 5 min in the control respiration buffer, mean state 3 respiration was 86 ± 5 ng of O/min/mg of protein, and mean state 4 respiration was 18 ± 3 ng of O/min/mg of protein (Fig. 2). The reduction in state 3 respiration is typical for isolated brain mitochondria after incubation periods at 37°C. When brain mitochondria were incubated for 5 min in respiration buffer containing DA (100 μ*M*), state 3 respiration was reduced by 24% as compared with respiration after incubation in buffer alone (Fig. 2). However, state 4 respiration was unaffected by the presence of DA (Fig. 2). To determine whether the

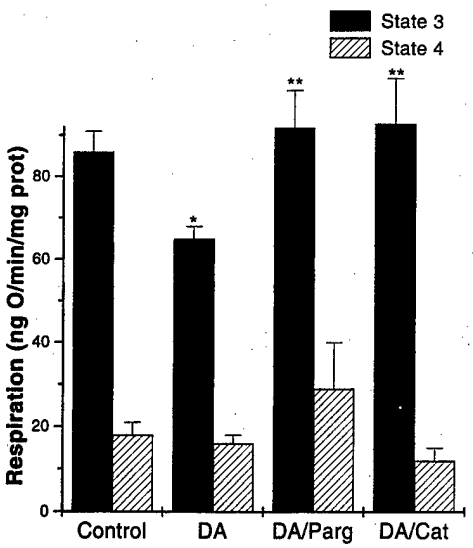

FIG. 2. Effect of DA on mitochondrial respiration. Isolated brain mitochondria were incubated for 5 min as described in Materials and Methods in buffer alone or buffer containing DA (100 μM), DA + pargyline (Parg; 10 μM), or DA + catalase (Cat; 1 U/ml), and then oxygen consumption was measured, utilizing glutamate and malate as substrates. Under control conditions, mean state 3 respiration was 86 ± 5 ng of O/min/mg of protein, and mean state 4 respiration was 18 ± 3 ng of O/min/mg of protein. *Significantly less than respiration under control conditions; **significantly greater than respiration after exposure to DA alone (mean ± SEM; n = 5–16).

state 3 inhibition caused by DA was due to H_2O_2 formed during MAO-catalyzed oxidation of DA, we examined the effects of adding the nonselective MAO inhibitor pargyline (10 μM) or the H_2O_2-scavenging enzyme catalase (1 U/ml) to the incubation medium. We found that the addition of either pargyline or catalase during the incubation with DA completely prevented the inhibition of state 3 respiration (Fig. 2). Neither pargyline nor catalase alone had any effect on mitochondrial respiration (data not shown).

To begin to determine which enzyme complexes of the electron transport chain were affected by DA, we utilized various substrates that donate electrons at different points in the electron transport chain, thereby hoping to bypass the DA-induced inhibition. We observed that when succinate and rotenone were utilized to bypass complex I and donate electrons via complex II to complex III, the decrease in state 3 respiration following incubation with DA was still observed (Fig. 3). Under these conditions, state 3 respiration was inhibited by 37% in the presence of DA as compared with control, an inhibition that was completely prevented by pargyline. However, when ascorbate and TMPD were utilized to donate electrons directly to cytochrome *c*, bypassing complexes I and III, incubation with DA no longer had any effect on state 3 respiration as compared with incubation under the same conditions without DA (Fig. 3).

To examine whether the products resulting from oxidation of the catechol ring would alter mitochondrial respiration, we utilized the enzyme tyrosinase to directly oxidize DA to DA quinone. Mitochondria were incubated for 5 min in standard respiration medium or medium containing DA (20 or 100 μM) and a concentration of tyrosinase (200 mU/ml) that was determined to oxidize all of the DA within 2 min (Berman and Hastings, 1997). Tyrosinase alone had no effect on respiration (data not shown). Following the incubation, we found that incubation with either concentration of DA quinone had no significant effect on state 3 respiration (Fig. 4A). However, we observed a dramatic increase in state 4 respiration with both the lower and the higher DA concentrations (+202 and +280%, respectively; Fig. 4B). Coincubation with GSH (500 μM), which can either reduce the DA quinone to DA or act as a nucleophile to bind DA, prevented the DA oxidation-induced increase in state 4 respiration at both concentrations of DA (Fig. 4B). GSH at this concentration had no effect on respiration (data not shown). When superoxide dismutase (SOD; 1 U/ml) and catalase (1 U/ml) were present, they did not prevent the increase in state 4 respiration caused by DA and tyrosinase (Fig. 4B).

Effects of DA oxidation products on mitochondrial permeability transition

Induction of permeability transition has been shown to lead to swelling of mitochondria (Gunter and Pfeiffer, 1990), which can be measured spectrophotometrically. Figure 5A shows the results of a representative swelling experiment. Exposure of brain mitochondria to control

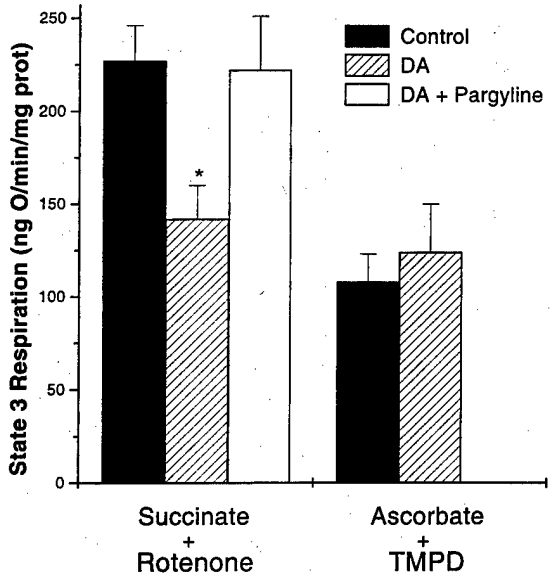

FIG. 3. Effect of bypassing steps in the electron transport chain on DA-induced inhibition of state 3 respiration. Mitochondria were incubated in buffer alone or buffer containing DA (100 μM) or DA + pargyline (10 μM) as described in Materials and Methods. Succinate (5 mM) + rotenone (2 μM) were utilized to donate electrons to complex II, and ascorbate (2 mM) + TMPD (100 μM) + antimycin A (1 μM) were utilized to donate electrons to cytochrome *c*. *Significantly less than respiration under control conditions (mean ± SEM; n = 3–8).

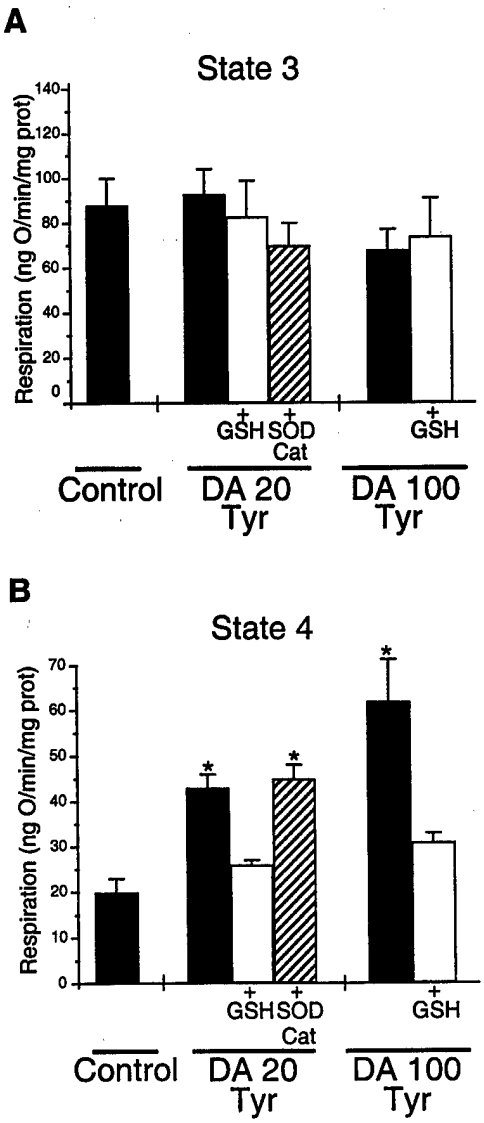

FIG. 4. Effect of enzymatic oxidation of DA on mitochondrial respiration. Mitochondria were incubated for 5 min in buffer or buffer containing DA (20 or 100 μM) + tyrosinase (Tyr; 200 U/ml) with or without GSH (500 μM) or SOD (1 U/ml) + catalase (Cat; 1 U/ml), and then respiration was measured using glutamate and malate as substrates. **A:** State 3 respiration. **B:** State 4 respiration. *Significantly different from control respiration (mean ± SEM; n = 3–13).

conditions (70 μM $CaCl_2$) led to a small degree of mitochondrial swelling. Exposure to 70 μM $CaCl_2$, followed by the addition of DA (100 μM), did not lead to an increase in the amount of mitochondrial swelling. However, exposure to the rapidly oxidizing catecholamine 6-OHDA (100 μM) significantly increased mitochondrial swelling. The results of these experiments, expressed as a change in absorbance, are quantified in Fig. 5B, demonstrating that 6-OHDA caused a threefold increase in mitochondrial swelling above control levels. CsA (850 nM), known to prevent the opening of the PTP in liver and heart mitochondria (Fournier et al., 1987; Crompton et al., 1988; Broekemeier et al., 1989), did not significantly prevent the swelling caused by 6-OHDA (Fig. 5B). CsA alone has no effect on swelling (data not shown).

We also examined the effects of DA quinone production using tyrosinase (Fig. 6). We found that brain mitochondria exposed to $CaCl_2$ (70 μM) followed by DA (100 μM) and tyrosinase (200 U/ml) led to a significant increase in mitochondrial swelling. In contrast to the findings with 6-OHDA, CsA (850 nM) was able to completely prevent the increase in swelling. Similar results were observed when a lower concentration of DA (20 μM) was utilized (Fig. 6). The addition of GSH (1

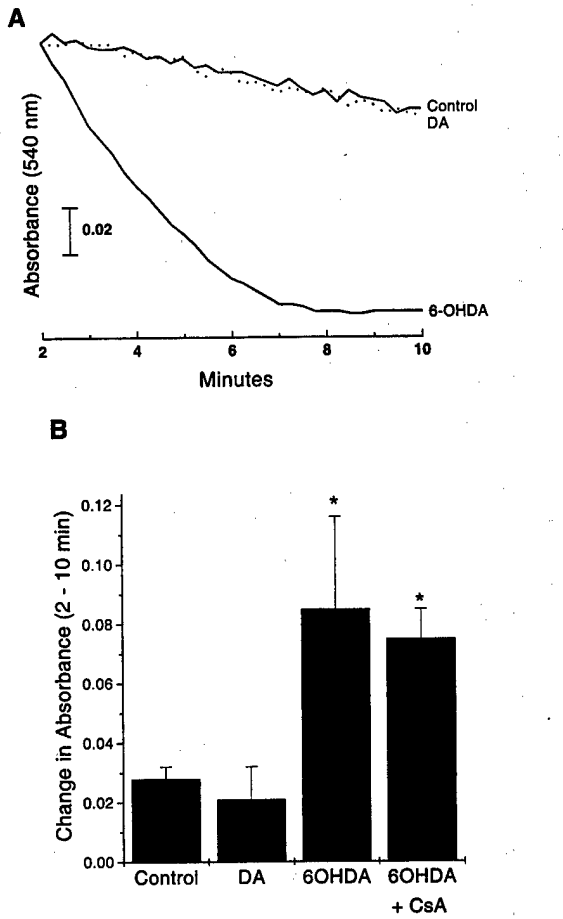

FIG. 5. Effect of DA and 6-OHDA on swelling of brain mitochondria. **A:** Representative traces of the change in absorbance at 540 nm, indicative of swelling in brain mitochondria, are shown following exposure to $CaCl_2$ (70 μM) alone (Control), $CaCl_2$ + DA (100 μM), or $CaCl_2$ + 6-OHDA (100 μM). $CaCl_2$ was added after 0.5 min, and DA or 6-OHDA was added at 2 min. When utilized, CsA (850 nM) was present at the beginning of the experiment. Absorbance changes due to the autoxidation of 6-OHDA were subtracted using blanks containing only buffer and 6-OHDA. **B:** Quantification of swelling measured as the absolute change in absorbance from the time the inducer was added (2 min) to 10 min (mean ± SEM; n = 3–6). *Significantly different from control values ($p < 0.05$).

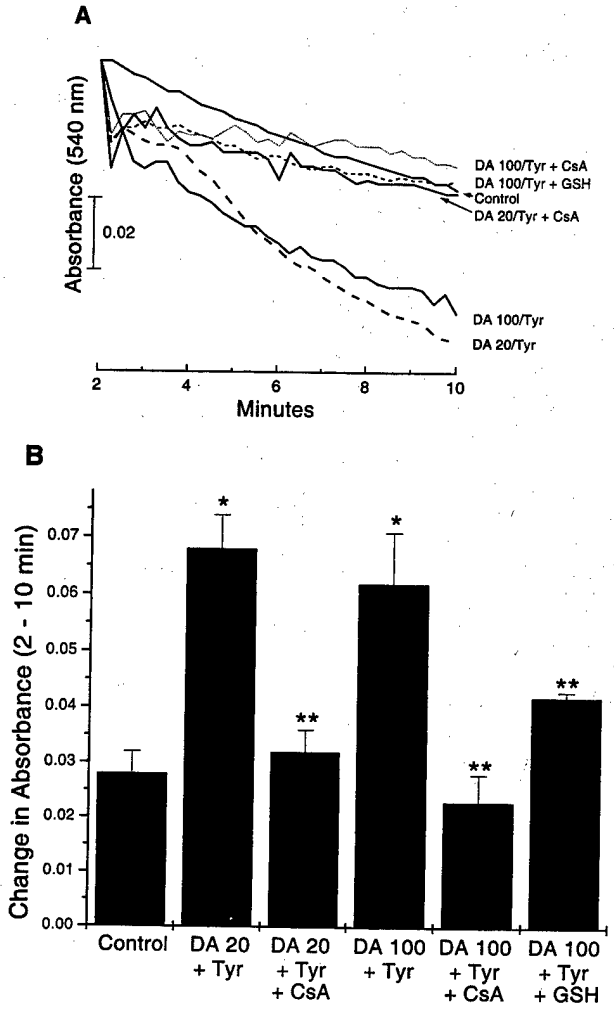

FIG. 6. Effect of tyrosinase (Tyr)-catalyzed oxidation of DA on swelling of brain mitochondria. **A:** Representative traces of swelling after exposure to $CaCl_2$ (70 μM) alone (Control) or + DA and tyrosinase (200 U/ml), with or without the addition of GSH (1 mM) or CsA (850 nM). $CaCl_2$ was added after 30 s, and then DA and tyrosinase were added at 2 min. When utilized, CsA and GSH were present at the beginning of the experiment. **B:** Quantification of swelling, measured as described in Fig. 5 (mean ± SEM; n = 3–6). *Significantly different from control values; **significantly different from the same condition without CsA or GSH.

mM) also significantly reduced the amount of swelling induced by DA and tyrosinase to levels similar to controls (Fig. 6).

The degree of swelling induced by DA oxidation in brain mitochondria is much smaller than that classically observed in studies of inducers of the transition pore. Most compounds have been tested in liver mitochondria, and we have noted that brain mitochondria respond to a much smaller degree to classic inducers of the PTP than do liver mitochondria (S. B. Berman, S. C. Watson, and T. G. Hastings, unpublished data). Therefore, we also examined the effect of DA quinone production on liver mitochondria. We observed that exposure of liver mitochondria to $CaCl_2$ (70 μM) followed by DA (100 μM) and tyrosinase (200 U/ml) resulted in large-amplitude swelling that was also prevented by the addition of CsA (Fig. 7). The change in absorbance induced in liver mitochondria by DA and tyrosinase was 11-fold higher than that observed in brain mitochondria (Figs. 6B and 7B) and was of similar magnitude to that observed for classic inducers of the PTP in liver mitochondria (e.g., Savage et al., 1991; Bernardi et al., 1992; S. B. Berman, S. C. Watson, and T. G. Hastings, unpublished data).

DISCUSSION

In this study, we provide evidence that both oxidation of the DA catechol ring and MAO-catalyzed DA oxidation can negatively affect the function of isolated brain mitochondria. We found that exposure of brain mitochondria to DA oxidation products altered both state 3 and state 4 mitochondrial respiration and caused mito-

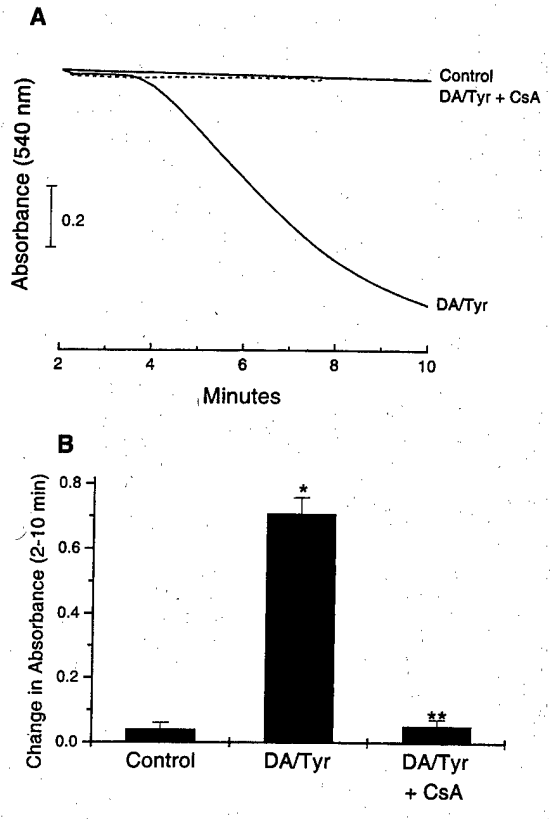

FIG. 7. Effect of tyrosinase (Tyr)-catalyzed oxidation of DA on swelling of liver mitochondria. **A:** Representative traces of swelling after exposure to $CaCl_2$ (70 μM) alone (Control) or + DA and tyrosinase (200 U/ml), with or without CsA (850 nM). Experiments were conducted as described in Fig. 6, except utilizing mitochondria isolated from liver. **B:** Quantification of swelling, measured as described in Fig. 5 (mean ± SEM; n = 3–6). *Significantly different from control values ($p < 0.05$); **significantly different from the same condition without CsA ($p < 0.05$). Note the different scale indicating absorbance change as compared with Fig. 6.

chondrial swelling that may be indicative of the opening of the PTP.

Mitochondrial respiration

DA oxidation to DA quinone. We provide the first evidence that oxidation of DA to DA quinone can lead to changes in mitochondrial respiration. We found that exposure of mitochondria to DA quinone resulted in a large increase in the resting rate of mitochondrial respiration (state 4). Normally, the transport of electrons through the electron transport chain is coupled to the pumping of protons from the matrix across the inner mitochondrial membrane, which is generally impermeable to protons (Brand et al., 1994). This establishes a proton gradient across the membrane, and protons are driven back into the matrix through the ATP synthase, providing the energy to convert ADP to ATP. In this way, mitochondrial respiration and its resultant oxygen consumption are coupled to ATP synthesis. If mitochondrial respiration were completely coupled to ATP synthesis, resting state 4 respiration would be zero, as ATP synthesis is not occurring. In healthy mitochondria, a low rate of respiration persists under state 4 conditions, and this is thought to be indicative of a low rate of proton leakage back across the inner membrane (Hafner et al., 1990). An increase in state 4 respiration therefore implies an increase in the proton leak across the membrane. After exposure to DA quinone, the rate of state 4 respiration increases nearly to the level of respiration measured in the presence of ADP (state 3). In other words, after exposure to DA quinone, respiration in the mitochondria proceeded without being coupled to ATP synthesis, an unproductive use of cellular energy.

It is possible that tyrosinase-catalyzed oxidation of DA produces not only the DA quinone but also $O_2^{\bullet -}$, albeit at a much lower level (Tomita et al., 1984; however, see Koga et al., 1992). To begin to determine whether the quinone is important in the effect on mitochondrial respiration that was observed, we investigated protection by either GSH or SOD plus catalase. GSH can reduce the quinone to DA or utilize its sulfhydryl group to covalently bind to DA quinone (Tse et al., 1976), thus scavenging the quinone and preventing it from binding to mitochondrial proteins. GSH was largely able to prevent the effect of DA and tyrosinase, suggesting that DA quinone is involved in the increase in resting respiration. SOD and catalase, which would detoxify any $O_2^{\bullet -}$ that might be formed, showed no protection against the effect caused by DA plus tyrosinase, further supporting the hypothesis that it is the DA quinone that is responsible for the uncoupling of respiration from ATP production.

Although the mechanism associated with the stimulation of resting respiration by DA quinone is not known, a similar effect on brain mitochondria was recently reported after exposure to the oxidant peroxynitrite (Brookes et al., 1998). This effect was prevented in the presence of the antioxidant Trolox. Although lipid peroxidation was suggested as a possible cause of the peroxynitrite effect, peroxynitrite, like DA quinone, can also modify sulfhydryl groups (Radi et al., 1991). Thus, it is also possible that covalent modification of sulfhydryl-containing proteins in the mitochondrial membrane contributes to the increase in proton permeability.

MAO-catalyzed oxidation of DA. We also found that MAO-catalyzed oxidation of DA, producing H_2O_2, led to an inhibition of active state 3 mitochondrial respiration. As both catalase and pargyline could prevent the inhibition, the production of H_2O_2 appears to be responsible for the effect. To determine where in the electron transport chain the inhibition was occurring, we utilized compounds that donate electrons directly to specific points along the chain, bypassing others. We found that inhibition of state 3 persisted when complex I was bypassed, suggesting that a process downstream of complex I is being affected. Furthermore, the inhibition disappeared when electrons were directly donated to cytochrome *c* just prior to complex IV, thus bypassing complex I and III. These findings suggest the possibility that the ubiquinone–complex III component of the electron transport chain is the target of the H_2O_2-induced inhibition. However, we cannot exclude the possibility that both complex I and II are inhibited by the H_2O_2. Both succinate dehydrogenase (complex II) and NADH dehydrogenase (complex I) have been shown to have thiol-dependent activity (Kenney, 1975; Benard and Balasubramanian, 1995) and thus may be susceptible to oxidation by H_2O_2.

This is the first study to examine the effect of DA on the oxygen consumption of well-coupled, healthy, intact mitochondria. Several previous studies examined only the effect of DA on complex I enzyme activity in tissue homogenates and reported conflicting results such as complete inhibition of complex I by 10 μM DA (Ben-Shachar et al., 1995), 25–50% inhibition with 1–100 m*M* DA (Przedborski et al., 1993), and 10% inhibition by 10 m*M* DA (Morikawa et al., 1996). The reasons for the discrepancies are not clear but may reflect different assay conditions. The two studies that began to investigate the role of DA oxidation in the enzyme inhibition noted prevention by antioxidants (Przedborski et al., 1993) and iron chelation (Ben-Shachar et al., 1995) but not by MAO inhibition (Ben-Shachar et al., 1995). All of these studies utilized only disrupted mitochondrial preparations to examine enzyme activities. However, studies utilizing intact, actively respiring mitochondria are likely to be more closely related to physiological circumstances, as the electron transport chain enzymes are normally coupled to ATP synthesis. In fact, studies have suggested that in nonsynaptic brain mitochondria, at least 70% inhibition of complex I is required before changes in state 3 respiration or ATP production are observed (Davey and Clark, 1996). Thus, the small inhibitory effect on respiration observed in our study may be indicative of larger changes in enzyme function. One other recent study utilized intact mitochondria but used extended incubation periods, and therefore less-coupled mitochondria, and utilized dye reduction rather than oxygen consumption as a measure of respiration, which does not allow examination of ATP synthesis-coupled

respiration (Cohen et al., 1997). It also studied only one of the two physiologic pathways of DA oxidation, MAO-catalyzed oxidation of DA, and reported an inhibition of respiration dependent on this oxidation, similar to our results. With avoidance of some of these limitations in our methods, the results of the current study confirm this finding as well as demonstrate significant effects on respiration by DA oxidation to DA quinone.

Mitochondrial permeability transition

Opening of the mitochondrial PTP has been suggested to play a critical role in several forms of neuronal cell death, including excitotoxicity, ischemia, MPP^+-induced toxicity, and hypoglycemia (Uchino et al., 1995; Nieminen et al., 1996; Packer et al., 1996; Schinder et al., 1996; White and Reynolds, 1996; Ouyang et al., 1997; Friberg et al., 1998). The increase in inner mitochondrial membrane permeability that is induced by PTP opening leads to mitochondrial membrane depolarization, release of small solutes and proteins, osmotic swelling, and a loss of oxidative phosphorylation (see Gunter and Pfeiffer, 1990; Bernardi, 1995). The PTP has been shown to contain critical thiol residues (Chernyak and Bernardi, 1996), and it has been induced by many oxidants including quinones (Gunter and Pfeiffer, 1990; Henry and Wallace, 1995; Henry et al., 1995). Therefore, we also examined effects of DA oxidation products on the PTP in mitochondria, and we conclude that DA quinone production resulted in the opening of the mitochondrial PTP. Mitochondrial swelling was induced in brain mitochondria after exposure to DA quinone that was completely prevented by the presence of the PTP inhibitor CsA, suggesting involvement of the PTP. GSH, which can inhibit DA quinone from reacting with sulfhydryl-containing mitochondrial proteins by covalently binding it as well as reducing it, was also able to prevent the swelling induced by DA quinone production.

Classically, most studies of the pore are performed in liver mitochondria, where a robust response has been well characterized (see Bernardi, 1995). To further investigate the effect of DA quinone production on classic pore characteristics, we also examined liver mitochondria. We found that enzymatic oxidation of DA produced large-amplitude swelling of liver mitochondria that was completely prevented by CsA, similar to that observed with other known pore inducers (e.g., Broekemeier et al., 1989; Bernardi et al., 1992). The magnitude of the swelling response in brain mitochondria was much smaller than that observed in liver (~10% of liver response) but is consistent with comparisons of PTP opening in brain and liver mitochondria after exposure to classic pore inducers such as calcium with phosphate (S. B. Berman, S. C. Watson, and T. G. Hastings, unpublished data).

The opening of the PTP induced by DA oxidation to DA quinone is interesting, given recent mechanistic studies of the transition pore. Evidence suggests that two sites exist on pore proteins that are important regulators of PTP function (Chernyak and Bernardi, 1996). One site contains vicinal thiols, which, when oxidized to disulfides, induce PTP opening. This site can be protected from oxidation by compounds that bind monothiols and prevent disulfide formation (Petronilli et al., 1994; Chernyak and Bernardi, 1996). One might expect that binding of DA quinone could protect similarly, binding directly to the monothiols. Benzoquinone has been suggested to inhibit PTP opening through this mechanism (Palmeira and Wallace, 1997). In contrast, there is also evidence that high concentrations of a monothiol-binding compound, *N*-ethylmaleimide, increased rather than decreased PTP opening (Petronilli et al., 1994), similar to the results with DA quinone.

A second important regulatory modulation involves the redox status of pyridine nucleotides. Oxidation of NADH and NADPH also increases pore opening, through an unknown mechanism that has been shown to be independent of the dithiol site (Chernyak and Bernardi, 1996). Oxidation of pyridines (both NADH and NADPH) can occur enzymatically through the cytosolic and mitochondrial enzyme DT-diaphorase, which reduces quinones via a two-electron reduction (Cadenas, 1995), and it has been shown that quinone substrates of DT-diaphorase can induce PTP opening (Chernyak and Bernardi, 1996). PTP opening by DA quinone could also be explained by this enzyme converting DA quinone to DA, oxidizing pyridine nucleotides in the process and increasing the probability of pore opening.

Availability of DA

For DA oxidation products to exert effects on mitochondrial function, DA must be available to mitochondrial proteins. Although the majority of DA in DA neurons is stored in vesicles, much of the DA clearly has access to mitochondria, because MAO, the major metabolizing enzyme of DA, is located on the outer mitochondrial membrane (Greenawalt and Schnaitman, 1970). One could hypothesize that under conditions of increased availability of cytoplasmic DA or increased synthesis and metabolism of DA, the potential for DA oxidation-induced effects on mitochondria would increase. Such conditions are thought to exist both in PD, where there is an increase in DA turnover (Bernheimer et al., 1973), and following high doses of methamphetamine, resulting in the redistribution of DA from vesicular storage to the cytoplasm (Cubbells et al., 1994; Sulzer et al., 1995). In fact, DA oxidation products have been shown to be increased in the substantia nigra of postmortem brain tissue from parkinsonian patients (Fornstedt et al., 1989; Spencer et al., 1998) and in rat striatum following exposure to methamphetamine (LaVoie and Hastings, 1999). Thus, these conditions may lead to an increase in cytoplasmic DA and subsequent increase in DA oxidation products, resulting in eventual dysfunction of mitochondria. Although this study was performed in vitro with isolated mitochondria and relatively high concentrations of DA, it raises the possibility that DA oxidation-induced alterations in mitochondrial function could occur under pathological conditions.

Conclusions

The alterations in mitochondrial function due to DA oxidation have several potential implications for neuronal cell death and neurodegenerative disease. As mentioned previously, it has been reported that individuals with PD exhibit a deficiency in the activity of complex I of the electron transport chain. It is not entirely clear whether this is a deficiency in all cells (Parker et al., 1989; Shoffner et al., 1991; Martin et al., 1996; Sheehan et al., 1997) or is limited to the substantia nigra (Schapira et al., 1990*b*; Mann et al., 1992), but the majority of evidence seems to point to a global deficiency. Therefore, the question arises as to the mechanism by which an underlying enzyme deficiency in all cells would lead to the specific loss of DA neurons. Our evidence suggests that one potential contributing factor may be the presence of DA. It is possible that an underlying deficiency, which alone does not cause cell death, is exacerbated by the presence of reactive DA metabolites. Other factors that have been implicated in PD, such as decreased antioxidant ability or increased iron (see Fahn and Cohen, 1992), also will contribute to increases in DA oxidation.

The effects of DA oxidation on mitochondrial function may also contribute to more acute neurotoxic events in which DA has been implicated. In methamphetamine toxicity, for example, DA is known to be important to the toxicity (Cubbels et al., 1994; Stephans and Yamamoto, 1994), and DA oxidation products have been shown to correlate with methamphetamine toxicity (LaVoie and Hastings, 1999). Thus, DA-induced mitochondrial dysfunction may also play a role in this neurotoxicity.

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THE ROLE OF THE PERMEABILITY TRANSITION IN GLUTAMATE-MEDIATED NEURONAL INJURY.

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THE ROLE OF THE PERMEABILITY TRANSITION IN GLUTAMATE-MEDIATED NEURONAL INJURY.

Reynolds and Hastings.

TABLE OF CONTENTS.

1. INTRODUCTION

Glutamate is the principal excitatory neurotransmitter in the brain. However, in addition to its critical role in fast excitatory neurotransmission, glutamate also has a more sinister role as a potent and effective neurotoxin, a process termed "excitotoxicity" (Rothman and Olney, 1987). It is well established that glutamate is acutely toxic to central neurons in primary culture, and it is widely believed that similar mechanisms contribute to the loss of neurons encountered in both acute and chronic neurodegenerative disease states. In the former category, there is good evidence that neuronal injury encountered in stroke, cerebral ischemia, traumatic brain injury and some forms of status epilepticus are mediated by excessive glutamate receptor activation. The links to chronic neurodegeneration are perhaps more tenuous. However, glutamate may mediate some part of the neurodegenerative process in amyotrophic lateral sclerosis, Huntingtons disease, Parkinsons disease, the dementia associated with acquired immunodeficiency syndrome, and possibly Alzheimers disease (see (Olney, 1990) for review).

The wide range of diseases associated with aberrant activation of glutamate receptors suggests that drugs that interrupt excitotoxicity could be of great therapeutic significance. Glutamate receptor antagonists have been intensively investigated in this regard, and have shown some promising results in acute disease models (Doble, 1999; Koroshetz and Moskowitz, 1996). However, this approach has been associated with significant behavioral side effects, reflecting the important role of glutamate in normal brain function (Tricklebank et al., 1987). An alternative approach to preventing excitotoxic injury could involve blocking some of the downstream processes that are activated by glutamate, and ideally would focus on a target that

was exclusively activated during injury, so that drugs directed towards this target should have much greater functional selectivity than glutamate receptor antagonists.

A number of recent studies that have investigated the mechanisms underlying excitotoxicity have identified a central role for mitochondria in the injury process. Moreover, it has been suggested that the permeability transition pore (PTP) is activated in neuronal mitochondria specifically during pathophysiological states, and that the activation contributes to the demise of neurons. If this is in fact true then the PTP should be considered a primary target for drug development in neurodegenerative disease. The goal of this review is to evaluate the validity of the conclusion that the PTP has a central role in neurodegeneration. To effectively address this issue, we will briefly review the characteristics of glutamate toxicity in acute and chronic disease states, and then consider the evidence for mitochondrial involvement in these processes. We will then evaluate the evidence for a role for PTP, as well as considering the limitations of the data supporting hypothesis that PTP is a critical event in the death of neurons.

2. THE ROLE OF MITOCHONDRIA IN GLUTAMATE TOXICITY

There is considerable evidence for a role for glutamate in acute brain injury. Glutamate is stored at high concentrations inside both neurons and astrocytes and is released into the extracellular space as a result of injury (Benveniste et al., 1984; Rothman, 1984; Strijbos et al., 1996). Selective glutamate receptor antagonists can ameliorate ischemic and traumatic injury *in vivo* (Gill et al., 1987; Boast et al., 1988). In addition, it is clear that glutamate is sufficiently effective as a neurotoxin that it can kill neurons *in vitro* without the need for any other injurious

agent (Rothman, 1984; Choi et al., 1987b). Thus, the study of the mechanism of action of glutamate should provide insights into some aspects of the process of neuronal injury, even though this approach undoubtedly takes an over-simplified view of the processes causing the death of neurons *in vivo*. It is also evident that glutamate-induced injury can take several forms, depending on the type of receptor that is activated (Koh et al., 1990; Mayer and Westbrook, 1987). The studies described here will focus on the most acute form of glutamate-triggered neuronal injury, namely that induced by the activation of N-methyl-D-aspartate (NMDA) receptors. It is clear that excessive activation of the other ionotropic glutamate receptors, the AMPA and kainate receptors, is also a highly effective way to kill neurons, but the cellular mechanisms responsible for injury are less well established.

2.1 Mitochondria in Neuronal Ca^{2+} Homeostasis.

Some of the earliest *in vitro* studies on glutamate toxicity demonstrated the requirement for extracellular Ca^{2+} for the expression of NMDA receptor mediated injury (Choi, 1987a). This observation is consistent with the Ca^{2+} permeability of the NMDA receptor associated ion channel (MacDermott et al., 1986). Moreover, the observation that extracellular Ca^{2+} decreases considerably during ischemia suggests a robust Ca^{2+} entry into neurons occurs *in vivo*, too (Erecinska and Silver, 1996; Kristián et al., 1994). The relationship between Ca^{2+} entry and neuronal death is still not entirely clear. It is only recently that it has been established that toxic stimulation of NMDA receptors actually results in larger intracellular free Ca^{2+} changes than the benign activation of other non-toxic Ca^{2+} elevating mechanisms (Hyrc et al., 1997; Stout and Reynolds, 1999). It has also been suggested that the route of Ca^{2+} entry is at least as important

as the magnitude of the influx, so that the precise location of a substantial Ca^{2+} influx may be the key to triggering injury (Tymianski et al., 1993; Sattler et al., 1998).

Studies of the mechanisms of Ca^{2+} homeostasis in neurons revealed an important role for mitochondrial Ca^{2+} transport following glutamate receptor activation (White and Reynolds, 1995; Khodorov et al., 1996; Wang and Thayer, 1996). We demonstrated that mitochondria and the plasma membrane Na^{+}/Ca^{2+} exchange (NCEp) both clear the cytoplasm of Ca^{2+} following a glutamate stimulus, and that the mitochondria account for a progressively larger proportion of the buffering ability as the intensity of the glutamate stimulus approached that which would ultimately prove toxic (White and Reynolds, 1995; White and Reynolds, 1997). A second approach to illustrating mitochondrial Ca^{2+} accumulation was the use of the mitochondrial NCE (NCEm) inhibitor CGP 37157 (Cox and Matlib, 1993). NCEm represents the predominant efflux pathway in functional neuronal mitochondria, so that blocking this pathway and monitoring the change in the recovery characteristics of the cytoplasmic Ca^{2+} concentrations allows the inference of a mitochondrial Ca^{2+} load (White and Reynolds, 1996; White and Reynolds, 1997; Baron and Thayer, 1997). Several studies have now reported the use of the Ca^{2+} indicator rhod-2 to illustrate mitochondrial Ca^{2+} loading in real time (Peng et al., 1998b; Peng and Greenamyre, 1998a; Minta et al., 1989), and these studies that measure Ca^{2+} changes in real time complement other approaches that have found glutamate- or injury-induced mitochondrial Ca^{2+} loading in isolated mitochondria (Sciamanna et al., 1992) or in brain slices using electron probe microanalysis (Taylor et al., 1999).

Without inferring the mechanism by which Ca^{2+} alters mitochondrial function, the importance of mitochondrial Ca^{2+} accumulation to neuronal injury has been illustrated by two

studies that blocked Ca^{2+} uptake during glutamate exposure and thereby prevented injury. Budd and Nicholls accomplished this in cerebellar granule cells using rotenone and oligomycin to depolarize mitochondria while maintaining intracellular ATP concentrations and were able to see protection against injury (Budd and Nicholls, 1996a; Budd and Nicholls, 1996b). We later used the protonophore FCCP and accomplished the same neuroprotection in forebrain neurons, although the reversibility of FCCP provided a better window of opportunity within which to observe the neuroprotection (Stout et al., 1998). These studies helped to establish the principle that mitochondrial Ca^{2+} uptake is essential for the expression of excitotoxicity, although the precise target of Ca^{2+} within mitochondria has not yet been established.

2.2 Reactive Oxygen Species and Glutamate Toxicity.

A number of studies have established that markers of oxidative stress are increased in association with ischemia and trauma, and in particular during the reperfusion phase following ischemia (Hall and Braughler, 1989; Phillis, 1994). There is also abundant evidence that chronic neurodegenerative states are associated with an increased oxidant burden (Götz et al., 1994). However, in most of these cases there are many potential sources of ROS (Halliwell, 1992) and the mechanisms responsible for the glutamate-specific alteration of the oxidant/antioxidant balance remain poorly defined.

A series of reports have detailed processes that could link glutamate receptor activation to ROS generation by mitochondria. Lafon-Cazal and colleagues (Lafon-Cazal et al., 1993) found that glutamate stimulated cerebellar granule cells generated ROS that could be detected by electron spin resonance. Dykens (Dykens, 1994) showed that isolated brain mitochondria

increased the generation of superoxide when presented with Ca^{2+} and Na^{+}, which is the circumstance presented to mitochondria in neurons activated by glutamate. Subsequently, we (Reynolds and Hastings, 1995) and others (Dugan et al., 1995; Bindokas et al., 1996) showed that glutamate-stimulated neurons in primary culture triggered ROS generation that could be detected with a range of oxidation-sensitive fluorescent dyes. A variety of arguments have been made to support a mitochondrial source for these effects. For example, one can infer from the location of the dye signal that oxidation occurs in the vicinity of mitochondria. This is not necessarily a compelling argument given the limited resolution with which these measurements can be made, and also the tendency of some of these dyes to distribute in cells to organelles that have no relation to their site of oxidation. Interrupting the ROS generating mechanism provides more persuasive insights into the source of ROS. We reported that the glutamate-stimulated effects on dichlorofluorescin oxidation were Ca^{2+} dependent, and that FCCP prevented glutamate-induced ROS generation (Reynolds and Hastings, 1995). Although FCCP has a number of effects on neurons (Tretter et al., 1998), the most straightforward basis for this inhibitory effect is the prevention of Ca^{2+} loading into mitochondria. It has also been shown that rotenone blocks ROS generation by dihydroethidium (Bindokas et al., 1996), which presumably is the result of inhibiting electron transport in Ca^{2+} -stimulated mitochondria. Together with the observation that oxygen-deprived neurons are resistant to glutamate-induced injury (Dubinsky et al., 1995), these findings suggest that the mitochondrially generated ROS may play a central role in excitotoxicity.

In addition to the ability of glutamate to directly induce ROS generation, there may be an additional interaction between oxidative stress and glutamate toxicity, in that an extrinsic

oxidative stress may potentiate the toxicity of glutamate. Thus, in neurons that are subjected to an oxidant burden the threshold for glutamate toxicity may be decreased (for example (Hoyt et al., 1997a)), and following oxidant inhibition of the glutamate transporter, the potency of glutamate may be increased (Piani et al., 1993; Volterra et al., 1994; Berman and Hastings, 1997). This may result in the increased vulnerability of selected populations of neurons. Moreover, it is possible that a critical target of the ROS is, in fact, the mitochondrion. Essentially all of the complexes in the electron transport chain are vulnerable to inhibition by oxidants (Zhang et al., 1990; Dykens, 1994; Berman and Hastings, 1999a), and the activities of several enzymes in the tricarboxylic acid cycle are impaired by oxidation (Chinopoulos et al., 1999). By further enhancing the generation of ROS, or by limiting the generation of ATP under circumstances that would normally place a great demand on the ATP supply (Chinopoulos et al., 1999), the impact of an oxidant burden together with glutamate exposure is potentially devastating.

One important example of this are the dopaminergic neurons in the substantia nigra that degenerate in Parkinsons disease. These neurons contain a high concentration of dopamine which generates an oxidant burden either by its metabolism by monoamine oxidase (MAO), which generates hydrogen peroxide, or by the formation of highly reactive dopamine quinones (Maker et al., 1981; Graham, 1978; Hastings, 1995). Dopamine is toxic to neurons in culture (Rosenberg, 1988; Hoyt et al., 1997b), and is also toxic when injected directly into the brain (Hastings et al., 1996). While the mechanism of this toxicity is not fully understood, a mitochondrial target is an important potential mechanism that is being evaluated. Thus, we have demonstrated that MAO-catalyzed oxidation of dopamine inhibits state 3 respiration in brain

mitochondria (Berman and Hastings, 1999a). Interestingly, dopamine quinone increased state 4 respiration, which would be consistent with an uncoupling effect perhaps attributable to an increase in membrane permeability associated with PTP activation (Berman and Hastings, 1999a). The oxidative burden associated with a high dopamine content and the concomitant bioenergetic impairment may render substantia nigra neurons especially vulnerable to excitotoxic stimuli (Greene and Greenamyre, 1996)

3. THE PERMEABILITY TRANSITION IN NEURONAL MITOCHONDRIA

The identification of mitochondria as a critical target for Ca^{2+} in neuronal injury moved the field one step closer to identifying the complete sequence of events necessary to execute the process of cell death. It seems likely that there are multiple mechanisms by which neurons may die following NMDA receptor activation (Ankarcrona et al., 1995), not all of which are associated with gross Ca^{2+} overload. Even when considering the acute, high Ca^{2+} load-associated injury, the precise target within mitochondria and the cellular consequences of altering this target have not been established. A simple view would be that neuronal death is simply a consequence of ATP loss, the failure of ion homeostasis and cell lysis that occurs as a result of uncontrolled solute accumulation. However, given that Ca^{2+} or ROS clearly do not shut down respiration or metabolism (indeed, Ca^{2+} stimulates ATP synthesis (McCormack et al., 1990)), what mechanisms are available to impair ATP synthesis under the circumstances generated by intense stimulation of NMDA receptors?

An attractive conceptual mechanism is provided by the permeability transition pore

(PTP). As a mitochondrial target that is activated by Ca^{2+}, oxidation and depolarization of the mitochondrial membrane, this target appears to be ideally suited as a point at which the principle effectors of neuronal injury can converge. We have already discussed the importance of mitochondrial Ca^{2+} accumulation and ROS generation. The third component, alteration of the mitochondrial membrane potential is then provided by the cycling of Ca^{2+} through mitochondria, which occurs at the expense of the proton gradient (Nicholls and Akerman, 1982; Gunter and Pfeiffer, 1990). Thus, the key activators of PTP should be present during glutamate exposure.

What evidence exists to support activation of PTP in the injury cascade triggered by glutamate? The key consequences of PTP activation should be a loss of membrane potential, alteration of mitochondrial shape, an increase in the permeability of mitochondria to small molecules, and, presumably neuronal death. Observations consistent with these events have now been reported by several laboratories. Several studies have found that mitochondria in intact neurons are depolarized during glutamate exposure using fluorescent dyes that report mitochondrial membrane potential (White and Reynolds, 1996; Schinder et al., 1996; Nieminen et al., 1996). The partial sensitivity of these changes in membrane potential to cyclosporin A is consistent with a contribution of PTP. Shape changes in mitochondria are difficult to resolve at the light microscopic level. Nevertheless, Ca^{2+} -stimulated, cyclosporin-sensitive changes in mitochondrial morphology have been reported, so that the predominant shape changed from rod-shaped to round in both neurons and astrocytes (Dubinsky and Levi, 1998; Kristal and Dubinsky, 1997). Recently, Friberg and colleagues established that the swelling of neuronal mitochondria in hypoglycemic brain injury can be prevented by cyclosporin A, thereby suggesting a link to PTP (Friberg et al., 1998). No studies in neurons have yet shown an explicit alteration in the

mitochondrial permeability of small molecules in intact neurons. However, several studies have found that neuronal death can be prevented by cyclosporin A, again consistent with an essential role for PTP in the death pathway (Schinder et al., 1996; White and Reynolds, 1996; Dawson et al., 1993). Although there are a number of potential limitations in these conclusions that will be discussed below, there is clearly plenty of evidence that could be interpreted as supporting a hypothesis that places transition as a final common pathway in the death of neurons.

4. LIMITATIONS IN THE PERMEABILITY TRANSITION HYPOTHESIS

Many features of the PTP are conceptually ideal in building a model of the glutamate-induced injury cascade. Indeed, it could be claimed that excitotoxicity represents the most clear example of the involvement of the PTP in a cellular or intact tissue injury paradigm. Nevertheless, it is obviously still necessary to apply the same critical standards to evaluating this hypothesis as any other. There are still a number of major issues that need to be more fully evaluated, and this section will examine the strengths and weaknesses of the PTP hypothesis as it is applied to neural cells.

4.1 Measuring Transition in Intact Cells.

The first major difficulty in evaluating the contribution of PTP to injury is measuring transition in intact cells. The study of transition has largely occurred in isolated mitochondria, and the most common approach currently used for assaying transition is the measurement of mitochondrial swelling using light scattering. A number of other approaches have been used,

including measuring the release of low molecular weight solutes such as glutathione, and also the influx of radioisotopes that are normally excluded from mitochondria with restricted permeability. However, the limitation in the ability to measure these parameters in cultured cells, because of the relative insensitivity of the methods, has thus far prevented their application in models of excitotoxicity.

As noted above, some recent studies have explicitly investigated the morphology of mitochondria in brains exposed to injury and have reported the prevention of the appearance of swollen mitochondria in response to hypoglycemia by cyclosporin A (Friberg et al., 1998). This is an exciting development in the study of PTP in neurons, but the approach does not lend itself well to mechanistic studies because of the difficulty in performing quantitative electron microscopic studies under circumstances where mitochondrial parameters can effectively be manipulated. Additional morphological approaches have been taken in permeabilized neurons and astrocytes. This is an interesting intermediate approach that falls between isolated mitochondria and intact cells. Dubinsky and colleagues were able to demonstrate Ca^{2+} mediated alterations in mitochondrial morphology, assayed in mitochondria loaded with fluorescent dyes, that could be partially prevented by the concomitant application of CsA (Kristal and Dubinsky, 1997; Dubinsky and Levi, 1998). These studies are also consistent with the activation of PTP in neural cells, although it remains difficult to be certain that the extent to which the environment presented to mitochondria in permeabilized or ionophore treated cells reflects the conditions created by glutamate exposure. Indeed, in astrocytes, that lack the efficient Ca^{2+} accumulation pathways found in neurons, the pathophysiological relevance of a Ca^{2+} overload-induced alteration in mitochondrial function remains to be established.

The application of morphological approaches to the study of mitochondrial shape in intact cells may also be limited by the dyes used to label mitochondria. Many of the mitochondrion-specific dyes accumulate in the organelle based on membrane potential, so that the change in membrane potential that should accompany the increase in permeability should grossly alter the dye staining properties (White and Reynolds, 1996). This could be an important confound when applying purely morphometric approaches. Other dyes, such as the series of MitoTracker™ dyes provided by Molecular Probes, may accumulate into mitochondria based on membrane potential, but then become irreversibly bound as a result of the interaction of the chloromethyl moiety of the dye with free sulfhydryls in (presumably) the mitochondrial matrix (Poot et al., 1996). While this provides the advantage of having a fluorescent marker that can be fixed, it has the important disadvantage of altering one of the important parameters that controls PTP activation, namely, the balance of reduced and oxidized sulfhydryls (Chernyak and Bernardi, 1996). The observation that MitoTracker orange can inhibit complex I with considerable potency, and can also activate transition in isolated hepatic mitochondria (P.Bernardi, personal communication) further underscore the difficulty of this approach.

Most other claims of PTP activation in neurons have been made based on cyclosporin-sensitive alterations in mitochondrial membrane potential (White and Reynolds, 1996; Schinder et al., 1996; Nieminen et al., 1996). These studies have typically reported an NMDA receptor stimulated, Ca^{2+} dependent depolarization of mitochondrial membrane potential using a range of potential-sensitive indicators. These depolarizations are generally observed during the time required to commit neurons to die as a result of the glutamate exposure, but occur well before the loss of viability can be detected, suggesting that the phenomenon is upstream in the injury

cascade. At least some of the time the depolarization is also reversible upon removal of glutamate (White and Reynolds, 1996), although this appears to be subject to some variability. It is obviously tempting to suggest that this loss of potential reflects transition, but is this conclusion reasonable? Based on the pharmacological evidence discussed below, where several putative transition inhibitors are effective this would appear to be a sound suggestion. However, it is difficult to exclude other possible mechanisms with confidence. Ca^{2+} is clearly essential in this process, but mitochondrial Ca^{2+} cycling occurs at the expense of the proton gradient (Nicholls and Akerman, 1982). Thus, the passage of a large amount of Ca^{2+} through mitochondrial uptake and release will presumably result in depolarization. It would also be difficult to distinguish between cycling-induced depolarization and a PTP triggered change because blocking Ca^{2+} uptake would block both phenomena concomitantly. Although Ca^{2+} cycling-induced mitochondrial depolarization has been observed in synaptosomes (Nicholls and Akerman, 1982), it has never been explicitly demonstrated in intact neurons. Nevertheless, its potential contribution is consistent with the observation of hyperpolarization of the mitochondrial membrane potential induced by the Ca^{2+} efflux inhibitor CGP 37157 (White and Reynolds, 1996). It is important to recognize that a depolarization of the mitochondrial membrane potential is also a normal response to an increased demand for ATP. Given that NMDA receptor activation will result in a substantial change in intracellular Na^+ as well as Ca^{2+} (Kiedrowski et al., 1994), and that the Na^+/K^+ ATPase is a major consumer of ATP in neurons, the greatly increased Na^+ burden should require an increase in ATP synthesis which should be accompanied by a depolarization of mitochondrial membrane potential. Thus, there are several major mechanisms that could produce an alteration in membrane potential that would be completely

independent of PTP, and, in fact, represent the function of normal, healthy mitochondria.

The intricate intertwining of PTP inducing stimuli and non-PTP related changes is further illustrated by the impact of oxidants in this system. In isolated mitochondria oxidants promote PTP activation, by oxidizing vicinal sulfhydryls or by increasing the pool of the oxidized form of glutathione (Chernyak and Bernardi, 1996). However, we also know that oxidants can inhibit electron transport, and may also limit the TCA cycle, which could alter the ability of mitochondria to pump protons and maintain a potential (Zhang et al., 1990; Chinopoulos et al., 1999). There is the additional confound of peroxide-induced changes in the properties of JC-1 that do not appear to be related to membrane potential (Scanlon and Reynolds, 1998; Chinopoulos et al., 1999) that can give the appearance of a depolarization, but which probably is not.

It is evident that the approaches used in intact neurons have not proved effective in unequivocally establishing the phenomenon of PTP activation. One potentially interesting approach that has not yet been applied to neurons is the cobalt-induced calcein quenching reported by Bernardi and colleagues (Petronilli et al., 1999), although it may prove difficult to apply this method to glutamate excitotoxicity models due to the interaction between cobalt and Ca^{2+} in this system. Approaches that combine morphology with membrane potential measurements, so that swelling can be observed in conjunction with a loss of membrane potential, together with a careful functional assessment, to preclude ATP synthesis and Ca^{2+} cycling as a cause of the membrane potential changes, may be necessary to definitively establish the expression of PTP activation in neurons.

4.2 Limitations in Pharmacological Approaches.

The difficulty in identifying PTP based solely on functional criteria in intact cells emphasizes the value of pharmacological intervention. Additionally, the putative contribution of PTP to glutamate-induced neuronal death might effectively be mitigated by effective antagonists of transition, so there is considerable interest in pore-specific drugs. Unfortunately, such agents are rather difficult to come by.

The classic PTP inhibitor is cyclosporin A (CsA). This agent binds to cylophilin D in the matrix and prevents the association of cylophilin with the pore complex and prevents the facilitative effect on PTP activation (Connern and Halestrap, 1994). CsA binds to cyclophilin with high affinity and with a specificity that is distinct from that associated with immunophilins (Bernardi et al., 1994; Connern and Halestrap, 1994). Thus, agents such as FK506 which are potent immunosuppressants do not alter transition in isolated brain mitochondria (Friberg et al., 1998). Conversely, analogues of CsA such as N-methylvaline-cyclosporin show PTP inhibition with rather less immunosuppressant activity (Griffiths and Halestrap, 1991). Unfortunately, these analogues are not commercially available. CsA is a less than ideal agent in intact neurons. The cyclic peptide structure may limit cell penetration, and studies in intact cells generally require higher concentrations that in isolated mitochondria. Some of the immunophilin-mediated effects, such as the inhibition of the Ca^{2+} -dependent phosphatase calcineurin, occur at lower concentrations that are apparently required for PTP inhibition (Dawson et al., 1993). Indeed, it has been suggested that calcineurin inhibition is neuroprotective independently of PTP, a suggestion supported by the neuroprotective effects of FK506 in some studies (Lu et al., 1996; Dawson et al., 1993) (but not others (Friberg et al., 1998)). The binding of CsA is also

modulated by Ca^{2+} and Ca^{2+} Mg^{2+}, with the effect that PTP inducing conditions may decrease the effectiveness of CsA binding (Novgorodov et al., 1994). This may explain the loss of effectiveness that is sometimes observed when CsA is used as an inhibitor of transition-associated events (Scanlon and Reynolds, 1998). There is no question that CsA is the most potent and probably the most useful of the putative pore inhibitors currently available. However, there are clearly other actions of CsA that are important for neuronal viability that are independent of cyclophilin D and PTP, and it is also true that CsA may not be effective even if PTP-mediated effects are under investigation. Combined with the difficulty in controlling intracellular CsA concentrations, there is a clear need for more effective inhibitors.

There are, in fact, a wide variety of agents that have been used to modulate PTP activity, both inhibitors and activators (see (Zoratti and Szabo, 1995)). These include atractyloside and bongkrekic acid, which bind to the ATP/ADP translocase. The suggestion that the PTP reflects a different functional state of the translocase is reflected in the ability of these agents to inhibit and promote PTP activation respectively (Halestrap and Davidson, 1990). Unfortunately, atractyloside is cell impermeant. The recent commercial availability of bongkrekic acid should allow its evaluation in neurons soon, although promoting PTP activation will obviously not effectively test the hypothesis that glutamate kills neurons following transition.

Some of the other agents that alter transition in isolated mitochondria have also been evaluated for their ability to block glutamate-mediated mitochondrial depolarization. Some of the more potent agents reviewed by Zoratti and Szabo (1995) include trifluoperazine and dibucaine. The former phenothiazine has a number of effects on cells, perhaps the most prominent of which are the inhibition of calmodulin and phospholipase activity. Dibucaine is

more widely known as a local anesthetic, an effect accomplished by the inhibition of voltage dependent Na^+ currents. Both of these agents delay the mitochondrial depolarization induced by glutamate, but were not entirely effective in preventing it (Hoyt et al., 1997c). In addition, both trifluoperazine and dibucaine appear to hyperpolarize mitochondria, while trifluoperazine may additionally inhibit mitochondrial Na^+/Ca^{2+} exchange (Hoyt et al., 1997c). We have previously observed a hyperpolarizing response to the NCEm inhibitor CGP 37157 (White and Reynolds, 1996). It is not clear whether this represents ongoing NCEm activity; the other possibility is a tonic activity of the PTP or perhaps a low conductance state of the pore (Ichas and Mazat, 1998). Given the limitations of the use of membrane potential for the unequivocal identification of transition making the distinction between these possibilities is difficult. However, that these drugs have multiple effects on mitochondria in addition to their other non-mitochondrial effects that can be expected to alter the function of excitable cells, illustrates the potential pharmacological limitations of these agents.

Some other drugs have recently been proposed to be PTP antagonists. The anti-estrogenic drug tamoxifen has been used as a pro-apoptotic agent in neural mitochondria at high concentrations (Ellerby et al., 1997). However, much lower concentrations prevented transition in hepatocytes (Custodio et al., 1998). We evaluated tamoxifen actions in neurons and found a biphasic effect on membrane potential changes induced by glutamate, with a maximum protection against depolarization observed at 0.3μM, much lower than the concentrations of tamoxifen required to injure the neurons. However, the maximally effective concentration of tamoxifen did not protect against excitotoxic injury (Hoyt, McLaughlin, Higgins and Reynolds, manuscript in preparation). Fontaine and colleagues recently reported effects of a series of

ubiquinone analogues on mitochondrial function in permeabilized skeletal muscle mitochondria (Fontaine et al., 1998). Several agents increased the ability of mitochondria to accumulate Ca^{2+} in a manner consistent with inhibition of transition. We evaluated these compounds in neurons with rather different results (Scanlon and Reynolds, manuscript in preparation). Thus, the most effective agent in muscle, Ub0, appeared to promote mitochondrial depolarization in neurons, and was quite toxic, likely as the result of ROS generation. Ub5 produced the greatest inhibition of depolarization, but did not protect against injury at all. It would be immensely valuable to have selective, potent and cell permeable PTP inhibitors in these studies, but it is clear that none of the currently known drugs have these properties.

4.3 Limitations in Cell Culture Methodology

In attempting to understand intracellular events associated with neuronal injury, there is clearly a great benefit in the use of cultured neurons, in that they are readily amenable to single cell study and to the kinds of manipulation that are often necessary to understand basic mechanisms. However, the value of the model system is only established by the fidelity by which it represents the situation *in vivo*. The value of the excitotoxicity model has been clearly established by the predictive ability of the system in identifying the neuroprotective actions of glutamate receptor antagonists that has subsequently been verified *in vivo*. However, there may be some important limitations of this model that have not been fully explored. For example, cells in culture may show a greater dependence on glycolysis rather than oxidative phosphorylation as a source of ATP. This could obviously have a profound impact on studies designed to link bioenergetic phenomena with glutamate stimulation. Neurons *in situ* have a

close and important interaction with astrocytes, which may be critical in the passage of nutrients from the cerebral circulation to neurons (Tsacopoulos and Magistretti, 1996). As the details of this interaction are poorly understood it is difficult to recapitulate this arrangement *in vitro*. Many imaging studies are performed at room temperature which may also have an important impact on the bioenergetic state of neurons.

There are important additional considerations in considering the value of cultured neurons. Many of the chronic neurodegenerative states are associated with the chronic, relatively modest inhibition of one or more of the complexes in the electron transport chain. Indeed, it is possible to model Huntington's and Parkinson's diseases using specific inhibitors of complex II and complex I respectively (see below). Acute disorders like stroke are associated with complete or partial restriction of oxygen and glucose. However, the studies that have investigated mitochondrial function following glutamate exposure have largely done so in an environment where oxygen and glucose are not limiting, and when electron transport is in good working order. Given that ROS generation, for example, may be increased under both hypoxic (Vanden Hoek et al., 1997) as well as presumably hyperoxic conditions, the study of states that more closely resemble actual diseases may be very important. There are clearly a multitude of potential influences on mitochondrial function, Ca^{2+} transport and ATP generation that need to be unraveled to improve the value of these model systems.

4.4 Acutely Isolated Mitochondria Preparations.

Much of what is known about the properties of mitochondria in the brain comes from studies of acutely isolated mitochondria, either in the form of purified organelles, or in the

context of synaptosomes, where the immediate environment surrounding the mitochondria is better preserved. Revisiting these findings is well beyond the scope of this review. Purified brain-derived mitochondria do exhibit a phenomenon similar to transition, in that mitochondria can be loaded with Ca^{2+} and exposed to oxidants in order to induce swelling (Andreyev et al., 1998; Friberg et al., 1999; Berman et al., 1999b). However, there are some important differences between the characteristics of activation of PTP in brain mitochondria compared to the more typically used liver preparations. For example, exposure of mitochondria to levels of oxidants and/or Ca^{2+} that would normally trigger swelling in liver mitochondria produces only small amplitude changes in mitochondria derived from brain, and the swelling is not accompanied by glutathione release as would be anticipated with PTP activation (Berman et al., 1999b). It is clear that this is not due to a lack of ability of brain mitochondria to swell, because higher amplitude swelling can be accomplished with mastoparan (Berman et al., 1999b), or by removing adenine nucleotides and Ca^{2+} Mg^{2+} (Andreyev et al., 1998; Friberg et al., 1999). However, the CsA sensitivity of the swelling that is observable is consistent with PTP playing a key role in the swelling process. Interpreting these differences remains a challenge, because an adenine nucleotide-free condition would not normally be found in neurons, and Ca^{2+} Mg^{2+} concentrations are near millimolar in these cells (Brocard et al., 1993). It has also been suggested that the polymerization state of creatine kinase in brain mitochondria could be a key difference from liver (O'Gorman et al., 1997). Nevertheless, the key point here is that it should not be assumed that the properties of PTP in brain are necessarily the same as the more completely characterized liver and heart preparations.

4.5 Mitochondrial Heterogeneity

The concept of transition is based largely, although certainly not entirely, on observations made in liver mitochondria. In trying to understand the contribution of PTP to glutamate induced injury to neurons many assumptions are based on the notion that the processes governing activation of PTP are the same in neural mitochondria as has previously been described in liver. Although there is little explicit information that points to functional differences in mitochondria that would account for different properties of the pore, it is not at all far-fetched to suggest that such differences exist. We know, for example, that there are differences in the efflux pathway for Ca^{2+} between liver mitochondria and those obtained from excitable cells (Gunter and Pfeiffer, 1990). It is also apparent that the fundamental properties of Ca^{2+} - and oxidant-induced swelling are distinct, as noted above (Berman et al., 1999b). We have also noted that oxidants such as *tert* butylhydroperoxide, which are highly effective pore-inducers in liver have very little effect on mitochondrial membrane potential in cultured neurons or isolated brain mitochondria (Scanlon and Reynolds, 1998; Berman et al., 1999b). This presents the need to approach expectations about the properties of the PTP in neurons in a rather cautious way.

An intriguing possibility is an additional level of heterogeneity between neurons and non-neuronal cells in the brain, between different populations of neurons or perhaps even between mitochondria in different regions of the same cell. Recent findings have demonstrated, for example, that the systemic administration of complex II inhibitors produces selective degeneration of striatal neurons in rats, which has proved to be a useful model for Huntington's disease (Beal et al., 1993). Remarkably, a similar approach using rotenone instead targets the dopamine terminals in the striatum followed by cell bodies in the substantia nigra, and producing

a syndrome in rats similar to Parkinson's disease (MacKenzie and Greenamyre, 1998). This raises the possibility that the mitochondria from different types of neuron in the same brain regions have distinct properties that results in their vulnerability to the different toxins. Another interpretation of these findings is that the mitochondria are, in fact, the same but the environment in which the mitochondria operate is different. For example, one might propose that the chronic exposure to high concentrations of dopamine make nigral neurons vulnerable to complex I inhibition, rather than a fundamental functional difference in the mitochondria. An argument in favor of this explanation was recently provided by an important study by Friberg and colleagues who demonstrated differences in the properties of PTP between mitochondria obtained from cortex, hippocampus and cerebellum (Friberg et al., 1998). However, these differences were apparently attributable to different concentrations of adenine nucleotides in the preparations rather than a fundamental difference in the mitochondria, because removal of the nucleotides resulted in the mitochondria exhibiting similar swelling characteristics.

The relevance of these findings to glutamate-induced neuronal injury have yet to be fully established. Many of the chronic disease states that can be modeled using electron transport inhibitors may also have an excitotoxic component, and an interaction between bioenergetics and the vulnerability to excitotoxic injury is well established (Greene and Greenamyre, 1996). However, the extent to which this interaction depends on the activation of PTP remains completely unknown.

5. CONCLUSIONS

In this review we have established that glutamate can injure neurons in a way that is likely to be relevant to a number of acute neurodegenerative states that include stroke and head trauma. It is also likely that glutamate contributes to the degeneration of neurons in more chronic diseases too. Many studies have established a relationship between the bioenergetic state of neurons and their vulnerability to injury, while more recent investigations have placed mitochondria at the center of the cascade of events that link glutamate receptor activation to neuronal death. However, the key question posed at the start of this review concerned the role of PTP in this process, and this is much less clear. There are significant concerns in the interpretation of the studies that have suggested that transition occurs in intact neurons because of the difficulty in attributing alterations in membrane potential to pore activation, so that these studies are suggestive but not conclusive. Morphological approaches are also suggestive of pore involvement, but there are similar methodological concerns. It is evident that CsA is neuroprotective under certain circumstances, but there are multiple mechanisms by which this could occur, and FK506 sometimes has neuroprotective actions too. None of the other drugs that alter membrane potential have demonstrated neuroprotective effects at this time. Thus, the body of evidence to link PTP to excitotoxicity is suggestive and intriguing but still rather inconclusive.

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Isolated brain mitochondria do not readily undergo permeability transition: Biochemical and ultrastructural analysis

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Abbreviations used: CsA: cyclosporin A; PhAsO: phenylarsenoxide; PTP: permeability transition pore; tBOOH: tert-butylhydroperoxide

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ABSTRACT: Opening of the mitochondrial permeability transition pore has increasingly been implicated in excitotoxic, ischemic, and apoptotic cell death, as well as in several neurodegenerative disease processes. However, most studies relating the transition pore to neuronal death have either utilized liver mitochondria or based findings on assumptions about properties that were developed in liver. It has largely been assumed that properties in brain mitochondria are similar to liver mitochondria, but this critical assumption has never been tested. In this study, we directly compared isolated brain mitochondria with liver mitochondria, and we provide evidence that brain mitochondria do not readily undergo permeability transition upon exposure to conditions that rapidly induce the opening of the transition pore in liver mitochondria. Exposure of liver mitochondria to transition-inducing agents led to a large, cyclosporin A-inhibitable decrease in spectrophotometric absorbance, a loss of mitochondrial glutathione, and morphologic evidence of matrix swelling and disruption, as expected. However, we found that similarly treated brain mitochondria showed very little absorbance change and no loss of glutathione. The absence of response in brain was not simply due to structural limitations, since large-amplitude swelling and release of glutathione occurred when membrane pores unrelated to the transition pore were formed. Additionally, electron microscopy revealed that the majority of brain mitochondria appeared morphologically unchanged following treatment to induce permeability transition. These findings show that isolated brain mitochondria are more resistant to induction of permeability transition than mitochondria from other tissues.

Key Words: mitochondria, permeability transition, neurodegeneration, apoptosis, excitotoxicity, oxidative stress

Running Title: Permeability transition in brain mitochondria

The important role of mitochondria in normal cellular functioning has long been recognized, and not surprisingly, abnormalities in mitochondrial function are increasingly found to play a significant role in cell death. This has become of particular importance in the brain, where investigations into the mechanisms responsible for neurodegenerative diseases and neurotoxic events have begun to focus on the potential contributions of mitochondrial dysfunction (for review, see Bowling and Beal, 1995). Whether mitochondrial abnormalities play a causative role in neurodegenerative diseases has not yet been established. However, mitochondrial dysfunction *has* been causally associated with the mechanisms of two forms of neuronal cell death that have been implicated in several neurodegenerative diseases: excitotoxicity and apoptosis (Deckwerth and Johnson, 1993; Vayssiére et al., 1994; Zamzami et al., 1995a; Zamzami et al., 1995b; Petit et al., 1995; Liu et al., 1996; Schinder et al., 1996; Susin et al., 1996; White and Reynolds, 1996; Ellerby et al., 1997; Green and Reed, 1998; Reynolds et al., 1998).

Accumulating evidence suggests that the alterations in mitochondrial function following an excitotoxic or apoptotic insult may be linked to the opening of a proteinaceous pore in the inner mitochondrial membrane, the permeability transition pore (PTP) (White and Reynolds, 1996; Schinder et al., 1996; Zamzami et al., 1995b; Ellerby et al., 1997; Kantrow and Piantadosi, 1997; Green and Reed, 1998; Miller, 1998). The opening of the PTP, termed permeability transition, allows the normally impermeable inner membrane of mitochondria to become nonselectively permeable to solutes with a molecular mass of 1500 Daltons or less. This leads to mitochondrial membrane depolarization, release of small solutes and proteins, osmotic swelling, and a loss of oxidative phosphorylation (see Gunter and Pfeiffer, 1990; Bernardi, 1995). The apparent

pharmacological inhibition of pore opening by cyclosporin A (CsA) has been shown to protect against apoptosis (Zamzami et al., 1996), mitochondrial depolarization induced by glutamate receptor stimulation (White and Reynolds, 1996; Schinder et al., 1996), ischemia (Uchino et al., 1995), and hypoglycemia (Friberg et al., 1998). In addition to inhibition of the PTP, however, CsA has many other actions in cells (Snyder and Sabatini, 1995), which makes a definitive relationship between protection by CsA and the PTP difficult in whole cells or tissue.

Most of the work directly characterizing the properties of the PTP have been performed in isolated preparations of liver and heart mitochondria, where actions of CsA are less ambiguous (See Gunter and Pfeiffer, 1990 and references therein). Studies using isolated mitochondria to directly relate the transition pore to neuronal death, such as after exposure to the parkinsonian neurotoxin, 1-methyl-4-phenylpyridinium (Packer et al., 1996; Cassarino et al., 1998; Cassarino et al., 1999), have used liver mitochondria and assumed that PTP properties in brain were similar. In this study, we directly compared the effects of exposure to known inducers of the PTP in liver mitochondria to those in identically isolated brain mitochondria. Using three different measures of pore opening, we provide evidence that the majority of brain mitochondria do not undergo permeability transition after exposure to conditions that rapidly induce permeability transition in liver mitochondria. These findings present the possibility that regulatory processes in brain differ from those in liver mitochondria and have important implications for the study of the mechanisms involved in neuronal cell death.

MATERIALS AND METHODS

Mitochondrial Isolation. Brain mitochondria from male Sprague-Dawley rats (300-350g; Hilltop Laboratories, Scottdale, PA), were isolated by differential centrifugation using a medium containing 225 mM mannitol, 75 mM sucrose, 5 mM K-HEPES, 1 mg/ml BSA, and 1 mM EGTA (pH 7.4), according to the method of Rosenthal et al. (1987). This method uses 0.02% digitonin to free mitochondria from the synaptosomal fraction. Digitonin binds cholesterol and permeabilizes cell membranes such as those of synaptosomes, but has little effect on mitochondria, which contain less cholesterol than cellular membranes (e.g. Sims and Blass, 1986). In order to maintain identical conditions, liver mitochondria were isolated from rat liver (1.5-1.75 g tissue) using the exact procedure as that for brain mitochondria. Mitochondrial protein yields for a single rat, determined by the method of Bradford (1976), were approximately 8-12 mg protein for brain and 20-25 mg protein for liver.

To ensure that the preparation contained healthy, functioning mitochondria, mitochondrial respiration was measured prior to the start of experiments (Rosenthal et al., 1987). Respiration measurements were determined polarographically with a thermostatically controlled (37°C) Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) in medium containing 125 mM KCl, 2 mM K_2HPO_4, 1 mM $MgCl_2$, 5 mM K-Hepes (pH 7.0), 1 mM EGTA, 5 mM glutamate, and 5 mM malate. Mitochondria were only used if the ratios of State 3 respiration (using 0.25 mM ADP) to State 4 respiration (using 2 μg/ml oligomycin) were determined to be at least 7.5. Rates of respiration (ng O/min/mg protein) were similar in brain and liver mitochondria.

Mitochondrial Swelling. Mitochondrial swelling was measured spectrophotometrically by monitoring the decrease in absorbance at 540 nm over 10 min similar to previously described methods (Broekemeier et al., 1989). Mitochondria (1 mg protein) were incubated in 2 ml of media containing 213 mM mannitol, 70 mM sucrose, 3 mM Hepes (pH. 7.4), 10 mM succinate, and 1 μM rotenone. In studies of the transition pore, $CaCl_2$ (70 μM) was added after 30 s, and other indicated compounds were added at 2 min. When cyclosporin A (CsA) was used, it was added to the buffer prior to the addition of the mitochondria. Data were quantified and compared by calculating the total decrease in absorbance from 2 min (the time the indicated inducers were added) to 10 min. In studies utilizing mastoparan, it was added after 30 s, and data were quantified by calculating the total decrease in absorbance from 30 s to 10 min.

GSH Measurements. Mitochondria were incubated as described for the swelling measurements. After the 10 min incubation, mitochondria were re-isolated via centrifugation at 12,000 *g* for 10 min at 4°C. The supernatant was removed, and protein was precipitated from the mitochondrial pellet via sonication in 0.1N perchloric acid with 0.2 mM sodium bisulfite followed by centrifugation at 18,000 *g* for 10 min at 4°C. The resulting supernatants, containing GSH from inside the mitochondria, were stored at -70°C until the time of the GSH assay. Total GSH (oxidized and reduced) was measured via the enzyme-coupled spectrophotometric method of Griffith (1980).

Electron Microscopy.

Mitochondria were prepared for electron microscopy either directly after the final centrifugation of the isolation procedure or following treatment with $CaCl_2$ and Pi as described for swelling experiments, followed by centrifugation at 12,000 *g* for 10 min. Electron microscopy methods are well established and only will be discussed briefly. Mitochondrial pellets were prepared and fixed in 2.5% glutaraldehyde in PBS. Following fixation, the samples were cut into small (1 mm^3) cubes, post-fixed with 1% osmium tetroxide, dehydrated, and embedded in Epon. Sections were cut using a Reichert Ultracut E ultramicrotome (Leica, Deerfield, IL), mounted on grids, and double-stained with 2% uranyl acetate (7 min) and 1% lead citrate (3 min). Observation was with either a Jeol 100CXII or Jeol 1210 TEM (Peabody, MA). To quantify the proportion of mitochondria affected by treatment with $CaCl_2$ and Pi, mitochondrial profiles were counted from randomly selected images collected at 25,000. Negatives from the two populations were coded and mixed and examined. The mitochondrial profiles were assigned either a normal or aberrant morphology by an experienced microscopist (SCW).

Statistical Analysis. Analyses were performed by one-way ANOVA followed by Tukey's *post hoc* comparisons. A probability of $p < 0.05$ was considered significant. N-values reported refer to data obtained from separate experiments.

RESULTS

Mitochondrial Swelling. Induction of permeability transition has been shown to lead to swelling of mitochondria (Gunter and Pfeiffer, 1990), which can be measured spectrophotometrically. In this study, known inducers of the PTP were tested for their ability to cause mitochondrial swelling in liver and brain mitochondria isolated by identical procedures. Similar to previously reported studies (see Gunter and Pfeiffer, 1990), exposure of liver mitochondria to 70 μM $CaCl_2$, followed by the addition of the inducers, Pi (3 mM K_2HPO_4), phenylarsenoxide (PhAsO, 5 μM), or tert-butylhydroperoxide (tBOOH, 1 mM) led to large decreases in absorbance, indicative of mitochondrial swelling (Figure 1A). Cyclosporin A (CsA) has been shown to prevent the opening of the PTP in liver and heart mitochondria (Fournier et al., 1987; Crompton et al., 1988; Broekemeier et al., 1989). We also found that pretreatment of liver mitochondria with CsA (850 nM) largely prevented the swelling caused by the pore inducers (Fig. 1A). In contrast, exposure of brain mitochondria to the same compounds led to much smaller changes in absorbance (Fig. 1B), with less potent inhibition by CsA. Expansion of the scale, as shown in Figure 1C, clearly reveals that the inducers caused some changes in absorbance in brain mitochondria albeit much smaller than those in liver.

Table 1 shows the quantified results of these treatments. The change in absorbance from the time the inducing agent was added (2 min) until the end of the experiments (10 min) was determined for each condition in liver and brain mitochondria. All of the inducers tested led to a significantly larger absorbance change over time in liver mitochondria as compared to control conditions. CsA pretreatment significantly reduced the absorbance change after exposure to the

inducers, by 89% with Pi, 72% with PhAsO, and 74% with tBOOH, indicative of a decrease in the amount of swelling. Exposure to the inducers in brain resulted in changes in absorbance that were 10-14% of responses observed in liver. However, the small responses were still statistically significant for Pi and PhAsO, although not for exposure to tBOOH (Table 1). Effects of CsA pretreatment also differed in brain as compared to liver, preventing only 46% of the change in absorbance caused by Pi and 51% of the change caused by PhAsO (Table 1).

Increasing the mitochondrial calcium load either by the presence of CGP 37157, an inhibitor of sodium-dependent calcium efflux from mitochondria (Chiesi et al., 1988; Cox et al., 1993), or by exposure to higher concentrations of calcium did not increase the amount of swelling in brain mitochondria (data not shown). The results were also similar with the use of an alternative buffer system, the KCl-based respiration medium, with longer incubation times, and with combinations of inducers (data not shown).

Loss of GSH. To begin to determine whether the differences between liver and brain swelling were due to differences in pore function or simply in the swelling properties of the different types of mitochondria, a second measure of pore opening, loss of mitochondrial GSH, was investigated. GSH is a small molecule that is accumulated in the mitochondrial matrix, and the loss of GSH from the matrix has been shown to occur upon pore opening (Savage et al., 1991). Liver and brain mitochondria contain comparable amounts of GSH following isolation and exposure to control conditions containing only $CaCl_2$ (Fig. 2). We found that treatment of liver mitochondria with $CaCl_2$ (70 μM) followed by Pi (3 mM) resulted in a profound loss (-82%) of mitochondrial

GSH (Fig. 2). CsA (850 nM) was able to completely prevent this effect. In brain mitochondria, however, $CaCl_2$ and Pi did not result in any loss of GSH (Fig. 2). Incubation with $CaCl_2$ (70 μM) and CsA had no significant effect on GSH levels in either brain or liver mitochondria as compared to controls.

Effects of Mastoparan on Swelling and GSH Release.

As a positive control, we tested the effects of the peptide mastoparan on mitochondrial swelling and GSH release in liver and brain. Although at lower concentrations, mastoparan can induce the PTP (Pfeiffer et al., 1995), at higher concentrations, such as the concentration used in this study (20 μM), mastoparan produces pores in the mitochondrial membrane in a non-CsA-dependent manner, thought to be unrelated to PTP opening (Nicolay et al., 1994; Pfeiffer et al., 1995). We observed that mastoparan caused large-amplitude swelling in both liver and brain mitochondria (Figs. 3A and B). In liver, maximal change in absorbance approximated that induced by permeability transition, whereas in brain, the change in absorbance was approximately 3-fold greater than with presumed inducers of the pore and averaged 40% that of liver (Fig. 3B). In addition, mastoparan caused the complete loss of GSH from both brain and liver mitochondria. Levels of GSH in brain and liver mitochondria after control incubation as in the swelling experiments were 2.94 ± 0.17 and 3.03 ± 0.33 nmol/mg protein, respectively (mean ± SEM; n = 3). Levels of GSH following exposure to mastoparan (20 μM) were nondetectable in both tissues.

Electron Microscopy. Figure 4 shows the typical fine structural morphology of mitochondrial preparations used within this study. In untreated preparations of brain mitochondria (Panel 4A), the predominant morphology shows small mitochondria with numerous electron dense branching cristae (arrow). The only contaminant in these preparations is free synaptosomes (chevron). In approximately 10% of mitochondria, vesiculated cristae may be seen (arrowhead). Following exposure to $CaCl_2$ (70 μM) and Pi (3 mM) (Panel 4B), large numbers of normal brain mitochondria are still seen (arrow) as well as synaptosomes (arrowhead). However, mitochondria showing fragmentation and vesiculation of cristae (arrowhead) now represent a larger (quantitatively 45%) proportion of the mitochondrial fraction in these preparations. Liver mitochondria prepared in the same fashion show a somewhat different morphology (Panel 4C): the intracristal volume appears reduced when compared with the brain mitochondria, principally due to an increased volume fraction of matrix within the mitochondria. Apart from occasional free membrane derived from the endoplasmic reticulum, there is very little contamination of this preparation. Treatment with $CaCl_2$ and Pi completely disrupts the mitochondrial morphology (Panel 4D). Although discrete membrane bound profiles persist, no recognizable structures are present within the mitochondria apart from free protein aggregates.

DISCUSSION

Many studies in the literature have characterized properties associated with the PTP in isolated mitochondria. However, the vast majority of these studies have utilized mitochondria isolated from either liver or heart. Our study reports the novel observation that brain and liver mitochondria behave biochemically and morphologically different after exposure to agents that have previously been shown to induce permeability transition in liver mitochondria.

Using three different measures, we provide evidence that isolated brain mitochondria do not readily undergo permeability transition upon exposure to conditions that rapidly induce the opening of the PTP in liver mitochondria. Exposure to transition-inducing agents led to a large, CsA-inhibitable decrease in spectrophotometric absorbance, a loss of mitochondrial GSH, and morphologic evidence of matrix swelling and disruption in liver mitochondria, as has been reported previously (e.g., Savage et al., 1991; Bernardi et al., 1992; Savage and Reed, 1994). However, we found that similarly treated brain mitochondria showed very little absorbance change and no loss of GSH. The absence of these responses in brain was not simply due to structural limitations, since large-amplitude swelling and release of GSH were induced when membrane pores unrelated to the PTP were formed by high concentrations of mastoparan. As additional evidence, electron microscopy revealed that the majority of the brain mitochondria appeared morphologically unchanged following treatments to induce PTP. The morphological changes that did occur were more subtle and did not reflect the complete destruction of structure that was observed in liver mitochondria.

Our evidence suggests, then, that brain mitochondria do not undergo permeability transition

under the same conditions that have been well-characterized for liver mitochondria. The reason for this difference is not known, but several possible explanations exist. One possibility is that only a fraction of mitochondria in the brain preparation are able to undergo permeability transition. Although the degree of swelling in brain mitochondria after exposure to Pi or PhAsO was small compared to liver, approximately half of the swelling was prevented by CsA, suggesting that this portion may be due to the PTP. The accompanying release of GSH from the mitochondria that undergo transition would then be only a very small amount compared to that still remaining inside the intact mitochondria and therefore, may be difficult to detect. In addition, since mitochondria are able to take up GSH (Griffith and Meister, 1985; Kurosawa et al., 1990; Martensson et al., 1990), any released GSH could potentially be transported into the intact mitochondria, masking a small effect.

The heterogeneous brain preparation includes mitochondria from both glia and neurons, and it is not known which population of mitochondria might be more susceptible to PTP induction. It is possible that one cell type or cells from specific regions in brain contain mitochondria that will undergo permeability transition, while others do not. The proportion of mitochondria from glial and neuronal cells in our preparation is not definitively known, but it is likely that there is a substantial proportion from both cell types. The method utilized in our studies is similar to isolation procedures for brain mitochondria which isolate both glial and neuronal (non-synaptosomal) mitochondria (e.g. Clark and Nicklas, 1970). However, the current method adds digitonin to the synaptosomal/mitochondrial fraction which permeabilizes synaptosomal membranes, increasing the neuronal mitochondrial yield (Rosenthal et al., 1987). The overall

mitochondrial yield increases from approximately 3-4 mg mitochondrial protein/brain in standard procedures (e.g. Clark and Nicklas, 1970) to 8-10 mg mitochondrial protein/brain in this preparation. Thus, it is likely that there is a substantial population of neuronal mitochondria as well as glial mitochondria. It is interesting to speculate whether the small degree of swelling observed in our study reflects the population of mitochondria from specific regions of the brain or from specific cell types that are susceptible to degeneration via mechanisms suggested to involve the PTP.

It is also possible that differences in protein expression between liver and brain could be responsible for the differences observed in this study. For example, recent evidence suggests that creatine kinase, which is present in high amounts in brain but not liver mitochondria, may be an inhibitor of the PTP (Beutner et al., 1998). In addition, differential protein expression may affect environmental factors that are known to regulate the PTP, such as intramitochondrial pH, the oxidation state of pyridine nucleotides and GSH, calcium load, or matrix magnesium (Bernardi, 1995). For example, oxidized pyridine nucleotides are known to increase the probability of PTP opening (Chernyak and Bernardi, 1996), and evidence suggests that the ability of pyridine nucleotides to be oxidized may be lower in brain mitochondria than in other tissues, due to differences in peroxidase activity (Lötscher et al., 1979; Beatrice et al., 1984; Satrustegui and Richter, 1984).

Certainly, other differences in mitochondria from liver and brain have been identified. For example, liver and brain mitochondria use diverse mechanisms to transport calcium; calcium efflux from brain mitochondria is primarily through sodium-dependent transport, whereas liver mainly

utilizes a sodium-independent mechanism (Gunter and Pfeiffer, 1990). Likewise, different PTP characteristics have also been identified in other tissues. Heart mitochondria, for example, are less sensitive than liver to swelling induced by calcium alone, and require either higher concentrations of calcium or an additional inducer such as Pi (Palmer and Pfeiffer, 1981; Novgorodov et al., 1992). Brain mitochondria appear to be even less sensitive, since exposure to calcium with inducers, higher concentrations of calcium, calcium efflux blockade, or even combinations of several inducers over longer periods of time did not result in large-scale opening of the PTP.

Conclusions

This study is the first to directly examine pore properties in brain mitochondria and compare them to the properties that have previously been well-described in other tissues. Other studies examining swelling of isolated brain mitochondria as a measure of permeability transition did not directly or quantitatively compare swelling to that observed in liver mitochondria nor use other measures of PTP opening (Kristal and Dubinsky, 1997; Friberg et al., 1998). It is clear from our findings that considerable differences exist in measures of PTP properties between isolated brain and liver mitochondria, and that sensitivity to variations in mitochondria from one tissue to another is critical. This is important to note, since previously, it has been assumed that pore properties are similar, and studies of isolated liver mitochondria have been utilized to extrapolate to brain (Packer et al., 1996; Cassarino et al., 1998).

In addition, it is very likely that heterogeneity exists between glial and neuronal mitochondria, between mitochondria from different regions of the brain, or even within different regions of a

single neuron. With reports suggesting a potential role for the PTP in neuronal injury due to apoptosis (Zamzami et al., 1996), excitotoxicity (Nieminen et al., 1996; Schinder et al., 1996; White and Reynolds, 1996), ischemia (Uchino et al., 1995; Ouyang et al., 1997), dopamine-induced toxicity (Berman and Hastings, 1999), and the parkinsonian neurotoxin, 1-methyl-4-phenylpyridinium (Packer et al., 1996; Cassarino et al., 1998a; Cassarino et al., 1999), better characterization of the properties of the PTP specific to brain mitochondria is critical.

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FIGURE LEGENDS

Figure 1. Representative traces of mitochondrial swelling induced by various agents, assessed spectrophotometrically. $CaCl_2$ (70 μM) was added after 30 s, and other compounds were added at 2 min [phosphate (Pi; 3 mM), phenylarsenoxide (PhAsO; 5 μM), and tert-butylhydroperoxide (tBOOH; 1 mM)]. When used, cyclosporin A (CsA; 850 nM) was present at the beginning of the incubation. Control samples were exposed only to $CaCl_2$. **A.** Liver; **B.** Brain; **C.** Brain (expanded scale). Arrow indicates the time that the inducers were added.

Figure 2. GSH remaining in the mitochondria was measured following a 10 min incubation period (mean ± SEM; n = 3-9). $CaCl_2$ (70μM) was added to all samples after 30 s of incubation, and Pi (3 mM) was added where indicated after 2 min.

Figure 3. **A.** Representative traces of mitochondrial swelling induced by mastoparan (20 μM), added at 30 s compared to control. **B.** Quantification of absorbance changes calculated as the absolute change in absorbance from 30 s to 10 min (mean ± SEM; n =3-4).

Figure 4. Electron micrographs of mitochondria from brain (A and B) and liver (C and D), isolated and prepared as described in *Methods*. Mitochondria either were untreated (A and C) or were incubated with $CaCl_2$ (70 μM) and Pi (3 mM) for 10 min as described in Figure 2 (B and

D). In panels A and B, normal brain mitochondria (arrow), larger mitochondria with fragmented cristae (arrowhead), and a small amount of contaminating synaptosomes (chevron) are observed. In liver mitochondria (C and D), nearly all mitochondria appear normal when untreated (C) and are completely disrupted when treated (D). Bar = 0.5 μm.

Table 1. Quantification of mitochondrial swelling after exposure to permeability transition inducing agents.

	Liver		Brain	
Treatment*	n	Decrease in absorbance (540 nm)†	n	Decrease in absorbance (540 nm)†
Control	3	0.024 ± 0.003	8	0.032 ± 0.003
Pi (3 mM)	5	0.958 ± 0.014[a]	3	0.130 ± 0.012[a]
Pi (3 mM) + CsA	4	0.095 ± 0.019[b]	4	0.074 ± 0.006[b]
PhAsO (5 μM)	3	0.853 ± 0.069[a]	3	0.099 ± 0.007[a]
PhAsO (5 μM) + CsA	3	0.230 ± 0.030[b]	3	0.049 ± 0.002[b]
tBOOH (1 mM)	3	0.550 ± 0.150[a]	3	0.052 ± 0.003
tBOOH (1 mM) + CsA	3	0.143 ± 0.059[b]	2	0.024 ± 0.004

*Mitochondria were exposed to $CaCl_2$ (70 μM) at 30 s, followed by the indicated agents at 2 min. When utilized, CsA (850 nM) was present prior to the addition of mitochondria.

†Values are the absolute change in absorbance from the time the inducing agent was added (2 min) to 10 min (mean ± SEM).

[a] Significantly different than control values ($p < 0.05$).

[b] Significantly different than the same condition without CsA ($p < 0.05$).

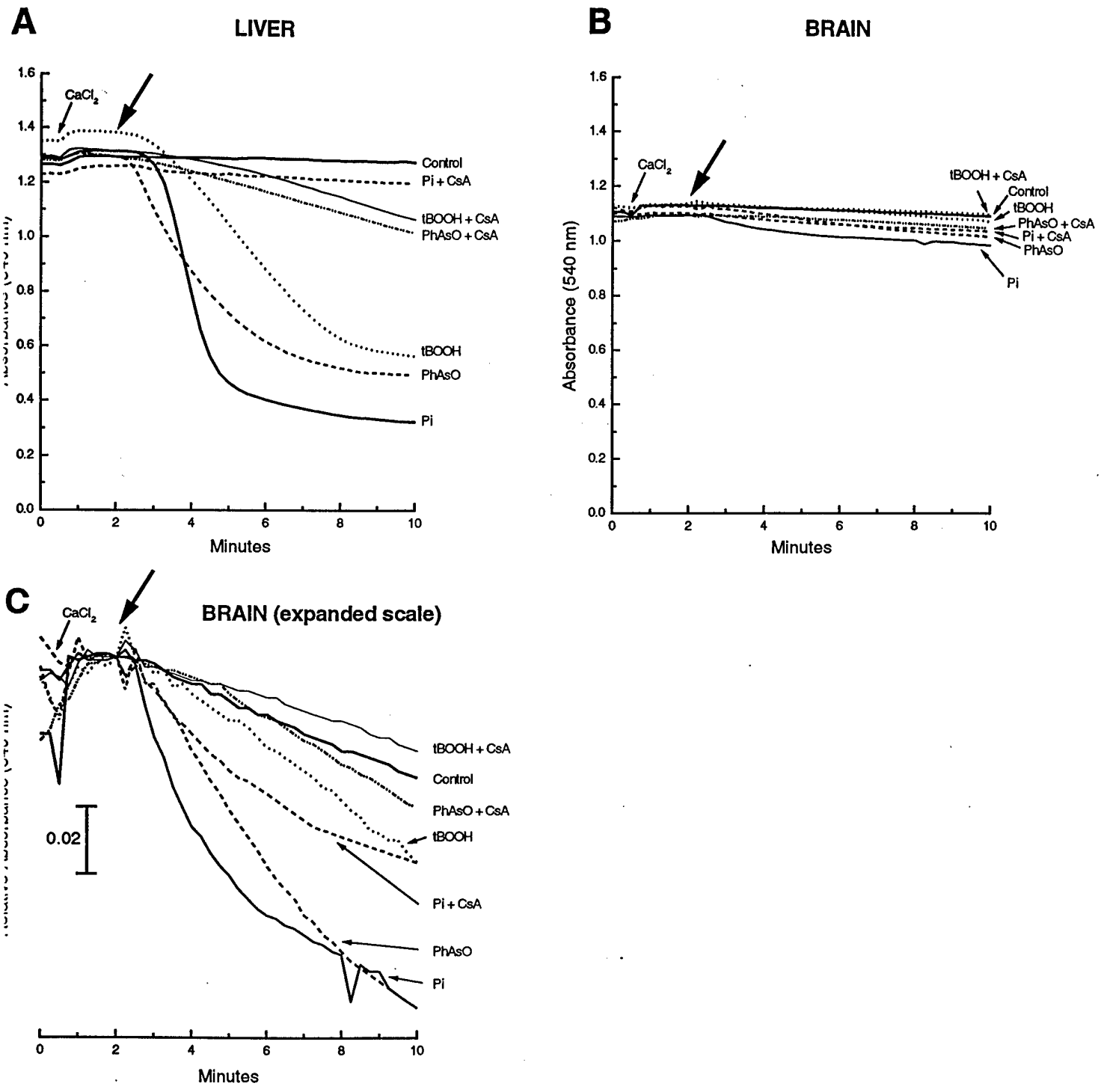

A
LIVER
CaCl2
Control
Pi + CsA
tBOOH + CsA
PhAsO + CsA
tBOOH
PhAsO
Pi
Minutes
B
BRAIN
CaCl2
tBOOH + CsA
Control
tBOOH
PhAsO + CsA
Pi + CsA
PhAsO
Pi
Absorbance (540 nm)
Minutes
C
BRAIN (expanded scale)
CaCl2
tBOOH + CsA
Control
PhAsO + CsA
tBOOH
Pi + CsA
PhAsO
Pi
0.02
Minutes

FIGURE 1

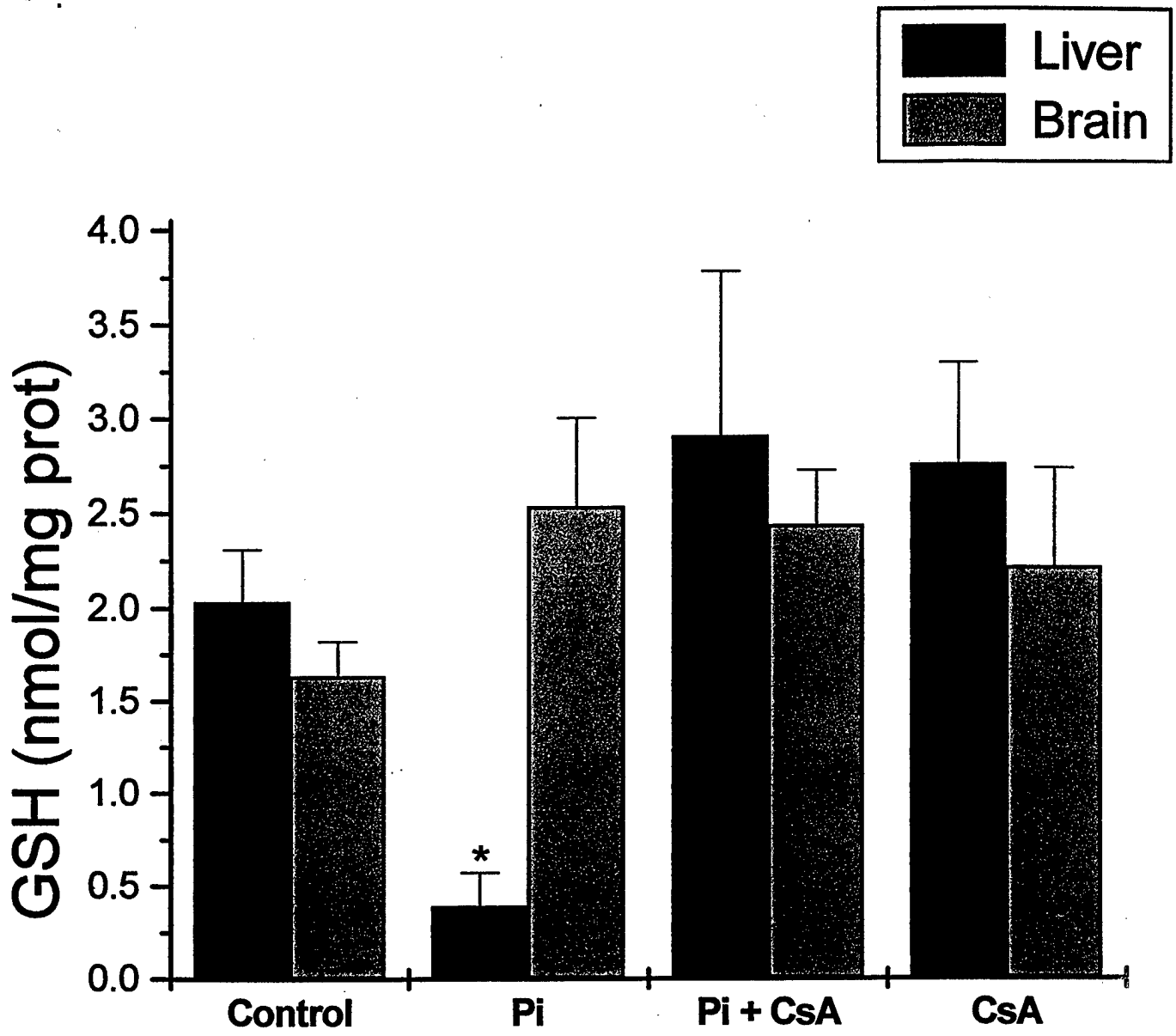
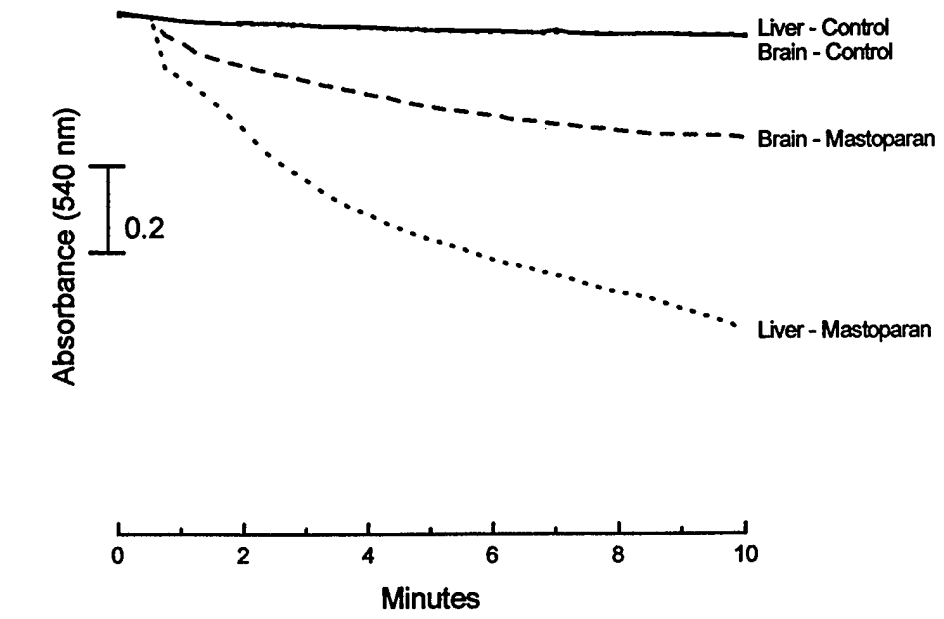

Liver
Brain
GSH (nmol/mg prot)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Pi
Pi + CsA
CsA

FIGURE 2

FIGURE 3


A
Liver - Control
Brain - Control
Brain - Mastoparan
Liver - Mastoparan
Absorbance (540 nm)
0.2
0
2
4
6
8
10
Minutes

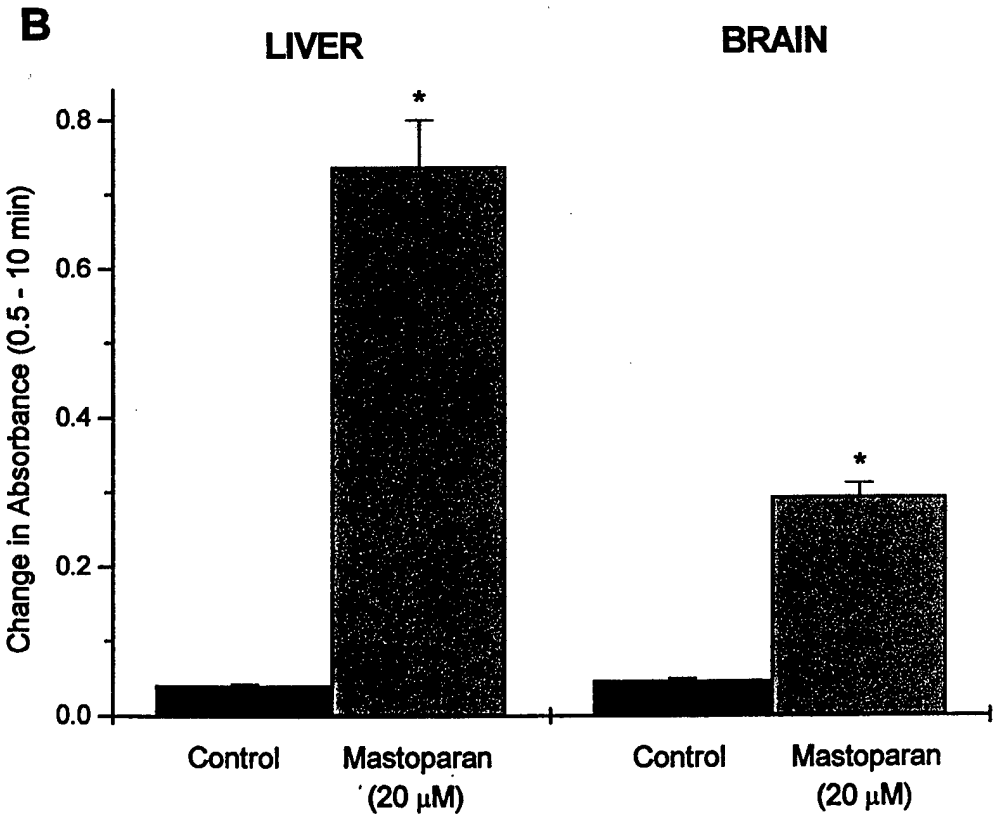
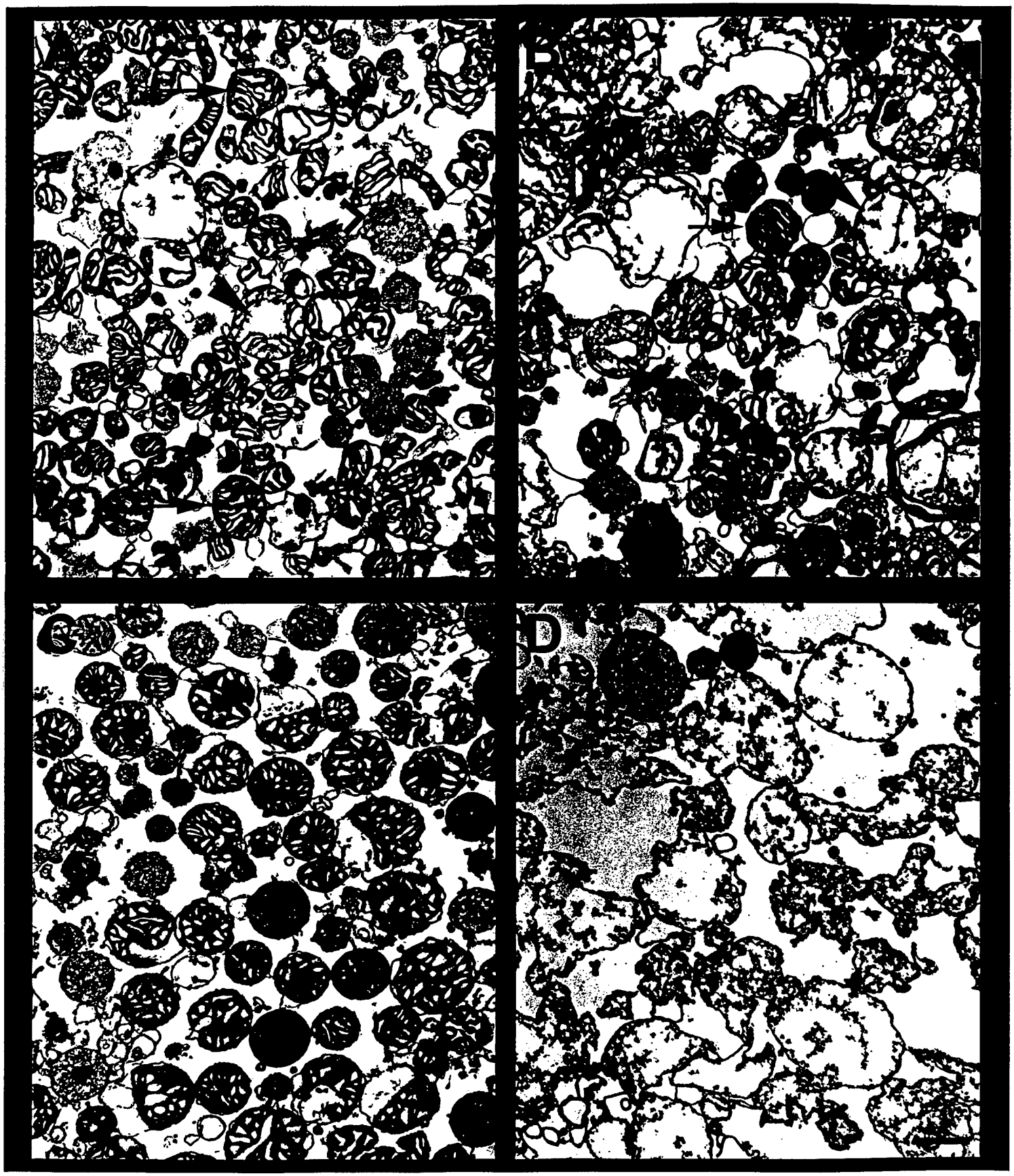

B
LIVER
BRAIN
Change in Absorbance (0.5 - 10 min)
0.0
0.2
0.4
0.6
0.8
*
*
Control
Mastoparan
(20 μM)
Control
Mastoparan
(20 μM)

FIGURE 4



Inhibition of glutamate-induced mitochondrial depolarization by tamoxifen in cultured neurons.

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Number of words in Discussion = 820

Abbreviations = HBSS, HEPES buffered salt solution; LDH, lactate dehydrogenase; $\Delta\psi_m$, mitochondrial membrane potential; PTP, mitochondrial permeability transition pore.

Abstract

In central neurons, glutamate receptor activation causes massive calcium influx and induces a mitochondrial depolarization, which is partially blocked by cyclosporin A, suggesting a possible activation of the mitochondrial permeability transition pore (PTP) as a mechanism. It has been recently reported that tamoxifen (an anti-estrogen chemotherapeutic agent) blocks the PTP in isolated liver mitochondria, similar to cyclosporin A (*Tox. Appl. Pharmacol.* 152: 10-17, 1998). In this study, we tested whether tamoxifen inhibits the mitochondrial depolarization induced by glutamate receptor activation in intact cultured neurons loaded with the fluorescent dye, JC-1. JC-1 reports disruptions in mitochondrial membrane potential, which can be caused by PTP activation. We found that glutamate (100 μM for 10 min) causes a robust mitochondrial depolarization which is partially inhibited by tamoxifen. The maximum inhibitory concentration of tamoxifen was 0.3 μM, with concentrations higher and lower than 0.3 μM being less effective. However, while tamoxifen (0.3 μM) blocked glutamate-induced mitochondrial depolarization, it did not inhibit glutamate-induced neuronal death, in contrast to the PTP inhibitor cyclosporin A. A relatively high concentration of tamoxifen (100 μM) caused mitochondrial depolarization itself, and was neurotoxic. These data suggest that tamoxifen may be a potent inhibitor of the PTP in intact neurons. However, the lack of specificity of most PTP inhibitors, and the difficulty in measuring PTP in intact cells, preclude definite conclusions about the role of PTP in excitotoxic injury.

Activation of the mitochondrial permeability transition pore (PTP) has been identified as a possible common effector of the cell death of numerous cell types in response to both necrotic and apoptotic stimuli (Lemasters et al., 1997; Kroemer et al., 1998). This pore is located in the inner mitochondrial membrane and, when opened, allows mitochondrial constituents < 1.5 kD to cross the inner membrane. In isolated mitochondria this results in swelling, loss of the protonmotive force, and the loss of low molecular weight compounds like glutathione (Savage and Reed, 1994; Zoratti and Szabo, 1995). Increases in matrix Ca^{2+} and oxidant levels are important inducers of the PTP. Cyclosporin A is among the most potent inhibitors of the PTP (Broekemeier et al., 1989).

The PTP has been suggested to be involved in the neurotoxicity caused by over-activation of neuronal glutamate receptors (Nieminen et al., 1996; Schinder et al., 1996; White and Reynolds, 1996). Glutamate-induced neurotoxicity is involved in the cell loss caused by stroke and trauma, as well as chronic neurodegenerative diseases (Choi, 1988). Activation of the various subtypes of glutamate receptor leads to opening of an integral ion channel and influx of Na^{+}, and in the case of the NMDA subtype and certain AMPA/kainate subtypes, Ca^{2+} (Mayer and Westbrook, 1987). Robust Ca^{2+} accumulation and the subsequent mitochondrial Ca^{2+} loading is critical for the expression of NMDA receptor mediated injury, although the events that link mitochondrial Ca^{2+} changes to toxicity have not been firmly established (Budd and Nicholls, 1996; Stout et al., 1998). Reactive oxygen species are generated by mitochondria in response to NMDA receptor mediated Ca^{2+} influx (Dugan et al., 1995; Reynolds and Hastings, 1995; Bindokas et al., 1996). The massive Ca^{2+} loading caused by NMDA receptor activation also induces a Ca^{2+} dependent depolarization of the mitochondrial membrane potential ($\Delta\psi_m$) which

is partially blocked by the PTP inhibitor cyclosporin A (Ankarcrona et al., 1996; Schinder et al., 1996; White and Reynolds, 1996) as well as other PTP blockers trifluoperazine and dibucaine (Hoyt et al., 1997). Cyclosporin A also inhibits toxicity caused by glutamate receptor activation although this effect may be mediated by calcineurin inhibition rather than PTP activation (Dawson et al., 1993; Ankarcrona et al., 1996; Schinder et al., 1996; White and Reynolds, 1996). Indeed, it has proven difficult to establish the role of the PTP in excitotoxicity because of the lack of potent and selective inhibitors.

It has been recently reported that tamoxifen, a widely-used anti-estrogen chemotherapeutic and chemoprevention agent, blocks Ca^{2+} -induced PTP activation in isolated liver mitochondria, with effects similar to cyclosporin A (Custodio et al., 1998). In addition to its' estrogen receptor blocking effects, tamoxifen is a lipophilic peroxyl radical scavenger (Custodio et al., 1994). However, it does not appear that its anti-oxidant function is related to its ability to block PTP since the PTP-inducing conditions (Ca^{2+} and phosphate treatment) with which tamoxifen was tested did not alter mitochondrial oxidized glutathione levels (an indication of oxidation) (Custodio et al., 1998).

Tamoxifen rapidly induces apoptosis in neural cell lines (Ellerby et al., 1997; Hashimoto et al., 1997). Whole cell extracts from cultures treated with 100 μM tamoxifen induced assymetric chromatin formations indicative of apoptosis in naïve isolated nuclei within 1 hour. This rapid morphological change was accompanied by caspase cleavage of nuclear substrates (Ellerby et al., 1997). These effects were not blocked by inhibitors of caspases 1 and 4, and could not be reproduced if nuclei were treated with only mitochondrial and cytosolic fractions from tamoxifen-primed cells. This apparent requirement for cellular components other than the mitochondria and cytosol would suggest that high levels of tamoxifen do not initiate cell death

by directly impairing mitochondrial membrane potential although this hypothesis has not been directly tested. It also remains to be determined if much lower doses of this compound can provide neuroprotection by altering PTP activation in primary neuronal cultures.

There are relatively few drugs available to study PTP activation in intact cells, and we were interested to see if tamoxifen would be as effective in neurons as it is in isolated mitochondria. We tested whether tamoxifen inhibits the $\Delta\psi_m$ depolarization induced by glutamate receptor activation in cultured neurons. $\Delta\psi_m$ was monitored in neurons loaded with the $\Delta\psi_m$ sensitive fluorescent dye, JC-1, as an indirect indication of PTP activation, since PTP activation necessarily results in a loss of $\Delta\psi_m$. We also determined the effect of tamoxifen on glutamate-induced neuronal death, both *in vitro* and *in vivo*.

Methods

Primary Neuronal Culture

Forebrain neurons were cultured from embryonic day 17 Sprague-Dawley rat pups as described (White and Reynolds, 1995). Pregnant rats were deeply anaesthetized with diethyl ether and were not allowed to regain consciousness. Embryos were then taken and used to obtain forebrain neurons. All animal handling procedures for isolation of neurons for cell culture were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Brain tissue was dissociated with trypsin, and then plated on to poly-D-lysine coated glass coverslips at a density of 450,000 cells ml^{-1} in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 24 units ml^{-1} penicillin and 24 $\mu g\ ml^{-1}$ streptomycin. Twenty-four hours after plating, the media were removed and replaced with DMEM that contained horse serum in place of fetal bovine serum, and the coverslips were inverted to suppress glial proliferation. Neurons were kept in a 37°C, 5% CO_2 humidified incubator for 12-18 days until use. All recordings were made using a HEPES buffered salt solution (HBSS) which contained (mM): NaCl (137), KCl (5), $MgSO_4$ (0.9), $CaCl_2$ (1.4), $NaHCO_3$ (3), Na_2HPO_4 (0.6), KH_2PO_4 (0.4), glucose (5.6), and HEPES (20), pH adjusted to 7.4 with NaOH . All glutamate solutions contained 1 μM glycine. Tamoxifen was dissolved in methanol (less than or equal to 0.02 % final methanol concentration) and all control conditions contained 0.02% methanol.

Measurements of mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\psi_m$) was estimated in individual neurons loaded with the $\Delta\psi_m$ sensitive fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-

tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Eugene, OR) (White and Reynolds, 1996). Neurons were loaded with the JC-1 (3 μM) for 20 min at 37°C , rinsed with dye-free HBSS for 20 min at RT or 37°C as indicated, and then mounted in a recording chamber on the stage of an ACAS 570c laser scanning confocal microscope (Meridian Instruments, Okemos, MI). Fields of neurons were illuminated using the 488 nm line of an argon laser, and emission at 530 and 590 nm was monitored. Solution changes in this protocol were made by rapidly aspirating and replacing the contents of the recording chamber. The fluorescence emission wavelength of JC-1 depends on the aggregation of the JC-1 molecules which in turn depends on the $\Delta\psi_m$ (the greater $\Delta\psi_m$, the greater the aggregation) (Reers et al., 1991). By monitoring JC-1 fluorescence at 590 nm (aggregate) and 530 nm (monomer) one can assess relative changes in $\Delta\psi_m$. Ratio values were obtained by dividing the signal at 590 nm by the signal at 530 nm after background subtraction on a cell-by-cell basis and normalized to a starting value of 1 for comparison between cells. Using this approach, a decrease in the normalized ratio represents mitochondrial depolarization.

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ was measured from individual neurons loaded with the Ca^{2+} sensitive fluorescent dye, indo-1 (White and Reynolds, 1995). Neurons were rinsed with HBSS and then loaded with 5 μM indo-1 AM (Molecular Probes, Eugene, OR) in HBSS containing 5 mg/ml bovine serum albumin for 50 min at 37°C, and incubated in dye-free HBSS for a further 20 min at 37°C to allow for dye cleavage. Coverslips were then mounted in a recording chamber (volume = 1 ml) on the stage of a Nikon Diaphot microscope. Cells were illuminated at 350 nm with light from a 75W mercury arc lamp. Indo-1 emission was simultaneously monitored at 405 and 490 nm

using a dual photomultiplier system. Background subtracted ratios were converted to $[Ca^{2+}]_i$ using parameters from an *in situ* calibration.

In vitro toxicity assay

For neuronal viability experiments, coverslips were washed once in HBSS that had been prewarmed to 37°C, inverted, and transferred to new plates. Cells were then washed twice more in HBSS and incubated in toxin. Cells were exposed to glutamate (100 μM) and glycine (1 μM) or HBSS in the presence or absence of tamoxifen (0.3 μM) and returned to the incubator for 10 minutes. Glutamate exposure was terminated by washing cells twice with HBSS. After rinsing with HBSS, cells were maintained in the presence or absence of tamoxifen (0.3 μM) in Minimal Essential Medium (MEM) . For high dose tamoxifen experiments, cells were maintained in 100 μM tamoxifen in MEM. Neuronal viability was determined 18-20 hour later for all experiments by measuring lactate dehydrogenase release with an *in vitro* toxicology assay kit (Sigma, St. Louis, MO). Forty microliter samples of medium were assayed spectrophotometrically according to the manufacturers protocol, to obtain a measure of cytoplasmic lactate dehydrogenase released from dead and dying neurons (Hartnett et al., 1997). LDH results were confirmed qualitatively by visual inspection of the cells. Chromatin staining of tamoxifen treated cells was also performed as described (McLaughlin et al., 1998). After incubation with tamoxifen, the cultures were washed briefly with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (pH 7.4) for 5 min, and incubated in 5 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 10 min. Cells were then washed twice in PBS and mounted on glass slides. Fluorescence of stained chromatin was evaluated using a Nikon Diaphot fluorescence microscope.

Striatal malonate lesions

Male Sprague-Dawley rats (275-350 gms) were maintained in a 12h/12h (light/dark) cycle with free access to standard rat chow and water. All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and have been approved by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University. Rats were anesthetized with equithesin, then placed in a Kopf small animal stereotaxic apparatus. A midline incision was made and the confluence of the sagittal and coronal sutures was identified (bregma). Malonate (3 μmol in 2 μl 0.9 N NaCl) was administered via a 26 gauge Hamilton syringe at a rate of 0.2 μl/ min, at the following coordinates relative to bregma: 0.7 mm anterior, 2.8 mm lateral and 5.0 mm ventral. The needle remained in place for an additional 5 min to limit regurgitation up the needle tract. Tamoxifen or vehicle (DMSO) treatments were administered i.p. 2h before and 4h after malonate exposure. Seven days following malonate exposure all animals were euthanized with chloral hydrate (500 mg/kg) and rapid decapitation. The cranial contents were removed, coated with embedding matrix, frozen under powdered dry ice and stored at -70°C until sectioning.

Coronal sections (25 μm) were gathered at 250 μm intervals through the rostrocaudal extent of the striatum using a cryostat and were thaw mounted onto poly-lysine treated slides. Tissue sections were then processed for cytochrome oxidase histochemistry.

Cytochrome oxidase histochemistry

Sections were incubated in 100 mM sodium phosphate buffer (pH 7.4) with cytochrome c (10 μM) and 3,3' diaminobenzidine (1 mM) for 2 h at 37°C in the dark. Sections were post-fixed in 10% formalin (10 min), dehydrated in graded alcohol and coverslipped from xylene. Analysis

of striatal lesion volume of cytochrome oxidase stained sections was performed on a microcomputer based image analysis program (Imaging Research, St. Catherines, Ontario) using area standards to provide a calibration from which 3D volume (mm^3) of the lesioned striatum was estimated.

Results

Exposure of neurons to excitotoxic concentrations of glutamate (100 μM) causes a decrease in $\Delta\psi_m$ that can be monitored with the $\Delta\psi_m$ sensitive fluorescent probe JC-1. A decrease in the ratio of JC-1 fluorescence emission at 590 nm relative to the emission at 530 nm indicates $\Delta\psi_m$ depolarization (Figure 1a). We have previously shown this depolarization is mediated primarily by the NMDA subtype of glutamate receptor and is Ca^{2+} dependent (White and Reynolds, 1996). When tamoxifen (0.3 μM) was included during the glutamate exposure (Figure 1a), there was a notable attenuation of the $\Delta\psi_m$ depolarization caused by glutamate. A protonophore, FCCP, which collapses the $\Delta\psi_m$, was added at the end of the fluorescence recording, and demonstrates a small additional depolarization that was not affected by tamoxifen. A higher tamoxifen concentration (20 μM) did not inhibit glutamate-induced mitochondrial depolarization (Figure 1b). We tested a range of tamoxifen concentrations (0.001 – 20 μM) on the glutamate-induced $\Delta\psi_m$ depolarization as in Figure 1a and b. As an expression of the magnitude of the effect of tamoxifen, we took the difference between the mean normalized JC-1 ratios after 5 min of exposure to glutamate (100 μM) in the presence or absence of tamoxifen (Figure 1c). The inhibitory effect of tamoxifen on glutamate-induced $\Delta\psi_m$ depolarization was maximal at 0.3 μM. Tamoxifen was less effective at concentrations greater or less than 0.3 μM, suggesting an additional effect of higher tamoxifen concentrations on $\Delta\psi_m$.

We tested whether tamoxifen alone affected $\Delta\psi_m$ and found no effect of tamoxifen at lower concentrations (less than 1 μM), and an apparent increase in $\Delta\psi_m$ induced by higher tamoxifen concentrations (10 or 20 μM) (Figure 2a). A prolonged exposure to a relatively high concentration of tamoxifen (100 μM) resulted in an apparent $\Delta\psi_m$ hyperpolarization followed by

a marked depolarization (Figure 2b). The loss of membrane potential preceded the loss of plasma membrane integrity, which occurred about 30 – 40 minutes after application of tamoxifen (data not shown).

We tested whether tamoxifen inhibits glutamate receptor activity as a possible mechanism of its inhibition of glutamate-induced $\Delta\psi_m$ depolarization. Tamoxifen (0.3 μM) did not inhibit glutamate-induced increases in $[Ca^{2+}]_i$ measured in indo-1 loaded neurons indicating that tamoxifen does not directly inhibit glutamate receptor activation (Figure 3a). Specifically, the glutamate-induced (3 μM for 15 s) peak $[Ca^{2+}]_i$ increase was 2.1 ± 0.4 μM (n = 7 neurons) and 1.7 ± 0.2 μM in the presence of 0.3 μM tamoxifen (n= 7 neurons; not significantly different from control, Student's t-test). Tamoxifen (0.3 μM) also did not affect the rate of Ca^{2+} recovery from a longer more intense glutamate stimulus (100 μM for 5 min) (Figure 3c). The time required to recover to twice basal Ca^{2+} levels in Ca^{2+} free HBSS was 47.1 ± 9.5 min (n= 8 neurons) and 42.5 ± 8.3 min in the presence of 0.3 μM tamoxifen for the two minutes following glutamate exposure (n = 6 neurons; not significantly different from control, Student's t-test). Agents which alter mitochondrial and plasma membrane Ca^{2+} buffering mechanisms affect the rate of Ca^{2+} recovery after glutamate (White and Reynolds, 1995; White and Reynolds, 1997; Hoyt and Reynolds, 1998; Hoyt et al., 1998). The lack of effect of tamoxifen on $[Ca^{2+}]_i$ or on Ca^{2+} recovery suggests that it does not inhibit glutamate-induced $\Delta\psi_m$ depolarization because of major alterations in $[Ca^{2+}]_i$ handling in response to glutamate.

It has been proposed that PTP activation is involved in the neurotoxicity of glutamate receptor activation, so we were interested to see if tamoxifen had a neuroprotective action. Tamoxifen (0.3 μM; present during and after glutamate exposure) had no effect on the neuronal death caused by glutamate (100 μM for 10 min) as measured by LDH release from damaged

neurons into the media during the 20h after glutamate exposure (Figure 4a). Since tamoxifen has been reported to rapidly induce apoptosis in neural cell lines, we tested a higher concentration (100 μM) of tamoxifen alone on neuronal viability and found that a 30 min exposure resulted in significant cell loss expressed 20 hr later (Figure 4b). Continuous exposure of neurons to 100 μM tamoxifen for 3h also caused an increase in the number of apoptotic nuclei visualized with the fluorescent nuclear dye, Hoechst 33342, from 3% in controls to 23% for cells treated with tamoxifen, consistent with previous findings in a neural cell line (Ellerby et al., 1997). It appears, therefore, that a low concentration of tamoxifen does not protect cells from excitotoxic injury and that high concentrations of tamoxifen are neurotoxic to primary cultured neurons.

We also tested whether tamoxifen was neuroprotective in an *in vivo* model of excitotoxic neuronal death. Malonate, an inhibitor of succinate dehydrogenase, causes metabolic inhibition and neuronal damage when injected into the striatum (Figure 5a). Glutamate receptor antagonists inhibit this neuronal damage, reflecting an excitotoxic component of this neuronal injury (Greene and Greenamyre, 1995; Schulz et al., 1996). Tamoxifen (2 mg/kg, i.p. 2h before and 4h after striatal malonate injection) did not reduce the volume of the striatal lesion (Figure 5b). Doses of tamoxifen from 1 – 20 mg/kg were tested and none prevented the striatal damage caused by malonate (Figure 5c).

Discussion

We found that glutamate (100 μM) causes a robust mitochondrial depolarization which is partially inhibited by tamoxifen. The maximum inhibitory concentration of tamoxifen was 0.3 μM, with concentrations higher and lower than 0.3 μM being less effective. Tamoxifen (0.3 μM) did not inhibit glutamate receptor activated increases in intracellular Ca^{2+} suggesting that it does not directly inhibit receptor activation, nor does it appear to inhibit $[Ca^{2+}]_i$ buffering after a glutamate stimulus. Therefore a decrease in glutamate-induced $[Ca^{2+}]_i$ levels by tamoxifen is unlikely to explain the inhibitory effect of tamoxifen on mitochondrial $\Delta\psi_m$ depolarization.

Tamoxifen did not completely inhibit glutamate-induced $\Delta\psi_m$ depolarization. This is similar to what we have previously reported for other PTP inhibitors, namely cyclosporin A, trifluoperazine and dibucaine (White and Reynolds, 1996; Hoyt et al., 1997; Scanlon and Reynolds, 1998). This may be a matter of time of onset of action of the particular drug, or its duration of action. There are other Ca^{2+} -stimulated effects on mitochondria in addition to activation of the PTP that would result in dissipation of $\Delta\psi_m$. These include mitochondrial Ca^{2+} cycling (Nicholls and Akerman, 1982) as well as ATP synthesis. Since we are not measuring PTP activation directly, and are unable to do so as yet in intact neurons, we cannot differentiate between PTP activation and other direct effects of glutamate receptor activation on $\Delta\psi_m$. The numerous additional effects of these PTP inhibitors on other cellular signal transduction mechanisms such as calcineurin, calmodulin and protein kinase C complicate the interpretation of effects of these drugs (Levin and Weiss, 1979; Liu et al., 1991; Rowlands et al., 1995; Gundimeda et al., 1996).

The lack of inhibition of glutamate-induced depolarization by tamoxifen at higher concentrations is puzzling. It is possible that at lower concentrations, tamoxifen has a relatively

selective effect on glutamate-mediated $\Delta\psi_m$ depolarization, while at higher concentrations, its membrane disruptive effects interact with the glutamate-induced mitochondrial dysfunction, leading to a lack of inhibition at these tamoxifen concentrations. These higher tamoxifen concentrations caused an increase in $\Delta\psi_m$. It is possible that tamoxifen affects one of a number of mitochondrial functions which could result in hyperpolarization. Among these possibilities are inhibition of the mitochondrial Na^+/Ca^{2+} exchanger, the F_1F_o-ATPase or a direct ionophore effect similar to nigericin (White and Reynolds, 1996; Hoyt et al., 1997). These possible mechanisms remain to be tested. High micromolar concentrations of tamoxifen induce rapid apoptotic death in neural cell lines (a finding which we confirmed in our primary cultures) (Ellerby et al., 1997; Hashimoto et al., 1997). The inability of tamoxifen-primed mitochondria to initiate apoptosis in naïve cell extracts suggest that nuclear or cell membrane associated caspases mediate the major component of tamoxifen induced programmed cell death (Ellerby et al., 1997).

Cyclosporin A inhibits glutamate-induced neuronal death *in vitro*, although the interpretation of the mechanism of this neuroprotective effect is complicated by multiple effects cyclosporin A has on cellular function, including inhibition of PTP as well as calcineurin activation (Dawson et al., 1993; Ankarcrona et al., 1996; Schinder et al., 1996; White and Reynolds, 1996). Since tamoxifen inhibited glutamate-induced $\Delta\psi_m$ depolarization similar to cyclosporin A, we were interested to see if tamoxifen protected neurons from glutamate-induced injury. Tamoxifen did not inhibit glutamate-induced neuronal death suggesting that either tamoxifen is not as effective an inhibitor of PTP as is cyclosporin A or that PTP activation is not a major contributor to the death caused by glutamate and that other actions of cyclosporin A explain its neuroprotective effect. We also tested whether tamoxifen could lessen the neuronal injury caused by excitotoxic injury to the striatum in an intact animal. Tamoxifen was not an

effective inhibitor of striatal injury at the doses tested (1 –20 mg/kg). Clinical doses of tamoxifen in humans are 0.4 to 0.8 mg/kg causing an acute serum concentration of about 0.07 μM and chronic (after 3 months) steady-state concentrations of about 0.2 μM (1997). Since tamoxifen is very lipophilic it is likely that tissue concentrations are higher than the serum concentration. It is possible that a higher and more prolonged tamoxifen exposure than used here would be neuroprotective. The lack of effect in primary culture argued against further testing this *in vivo*.

These data suggest that tamoxifen may be a potent inhibitor of PTP in intact neurons, in agreement with previous studies in isolated liver mitochondria. However, given its lack of specificity, conclusions about the role of PTP in glutamate-induced mitochondrial depolarization are not yet possible and await the development of selective PTP inhibitors, as well as a reliable assay for PTP activation in intact cells. Since tamoxifen is already a widely-used drug for chemotherapy and chemoprevention, a potential neuroprotective effect would have been very useful clinically. However, under our conditions, tamoxifen was unable to inhibit relatively acute excitotoxic neurodegeneration. If inappropriate PTP activation is involved in the more chronic neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease or Huntington's disease, then inhibitors of PTP activation would ultimately be beneficial.

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Footnotes

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Figure Legends

Figure 1: Tamoxifen inhibits glutamate-induced mitochondrial depolarization in neurons loaded with JC-1. *A:* Application of 100 μM glutamate (closed squares) caused a decrease in the normalized 590 nm/530 nm JC-1 emission ratio, reflecting mitochondrial depolarization. Addition of 0.3 μM tamoxifen (open squares) during the glutamate exposure substantially reduced the extent of the loss of $\Delta\psi_m$ caused by glutamate. Data represent the mean ± s.e.m. of 54-70 neurons per condition. FCCP (750 nM), a protonophore which depolarizes $\Delta\psi_m$, was added at the end of the experiment for comparison. *B:* A higher tamoxifen concentration (20 μM) did not inhibit the glutamate-induced decrease in $\Delta\psi_m$, when included during the glutamate exposure. Data represent the mean ± s.e.m. of 51-61 neurons per condition. *C:* Concentration-dependence of the inhibition of glutamate-induced mitochondrial depolarization by tamoxifen. Data are expressed as the difference between the normalized JC-1 ratio for tamoxifen-treated vs. untreated cells after 5 min of glutamate exposure. The range of the s.e.m. for the datapoints from which the differences were calculated was 0.018 – 0.025 normalized JC-1 fluorescence units. As the tamoxifen concentration was increased, there was a decrease in the inhibitory effect on glutamate-induced depolarization. Data were collected from a total of 41-70 neurons.

Figure 2: Tamoxifen, at relatively high concentrations, increases the apparent $\Delta\psi_m$. *A:* A range of tamoxifen concentrations was tested on the $\Delta\psi_m$ in JC-1 loaded neurons. Concentrations of tamoxifen lower than 1 μM had little direct effect on $\Delta\psi_m$, while higher concentrations (greater than 10 μM) caused an increase in the JC-1 ratio, presumably reflecting an increase in $\Delta\psi_m$. Data represent the mean ± s.e.m. of 21-41 neurons per condition. *B:* A

prolonged exposure to tamoxifen (100 μM) causes an increase in the $\Delta\psi_m$ followed by a pronounced decrease in $\Delta\psi_m$. Data represent mean ± s.e.m. of 14 neurons from a single culture date, and are representative of data collected from a total of 3 experiments.

Figure 3: Tamoxifen did not inhibit glutamate-induced increases in $[Ca^{2+}]_i$. *A:* Indo-1 loaded neurons were exposed to 15 sec pulses of 3 μM glutamate/1 μM glycine (arrows). When tamoxifen (0.3 μM) was included prior to and during the glutamate stimulus, there was no alteration in the $[Ca^{2+}]_i$ increase induced by glutamate. Data are representative of Ca^{2+} traces collected from 7 neurons. *B:* Tamoxifen does not affect $[Ca^{2+}]_i$ recovery following a glutamate stimulus. Neurons were exposed to 100 μM glutamate/ 1 μM glycine for 5 min, and then immediately exposed to Ca^{2+} free HBSS or tamoxifen (0.3 μM) in Ca^{2+} free HBSS for 2 min immediately following glutamate exposure. Note that there is no apparent effect of tamoxifen on the rate or shape of the $[Ca^{2+}]_i$ recovery. Data are representative of Ca^{2+} traces collected from 5-7 additional neurons.

Figure 4: Effects of tamoxifen on neuronal viability and on excitotoxicity *in vitro*. *A*: Tamoxifen (0.3 μM) does not inhibit glutamate-induced neuronal death. Neurons were exposed to 100 μM glutamate for 10 min in the presence or absence of 0.3 μM tamoxifen, and neuronal death was assessed 20 h later using LDH release into the media as a measure of neuronal damage. * = $p < 0.01$, significantly different from untreated control, ANOVA with Bonferroni correction for multiple comparisons. *B*: A relatively high concentration of tamoxifen (100 μM) causes neuronal death. Neurons were exposed to 100 μM tamoxifen for 30 min, and LDH

release was assayed 20 h later. Data represent the mean ± s.e.m collected from at least three culture dates, * = $p < 0.01$, significantly different from untreated control, Student's t-test.

Figure 5: Tamoxifen does not inhibit formation of striatal lesions induced by malonate. *A:* Injection of malonate into the rat striatum induces a lesion that was visualized 7 days post-injection by staining for cytochrome oxidase. *B:* Treatment with 2 mg/kg tamoxifen 2h before and 4h after malonate injection did not decrease the size of the malonate-induced lesion. *C:* A range of tamoxifen doses (1 – 20 mg/kg) did not protect against striatal malonate lesion formation. Data represent the mean ± s.e.m. collected from 3-13 rats per condition.

Index Terms

tamoxifen, mitochondria, excitotoxicity, JC-1, glutamate, apoptosis

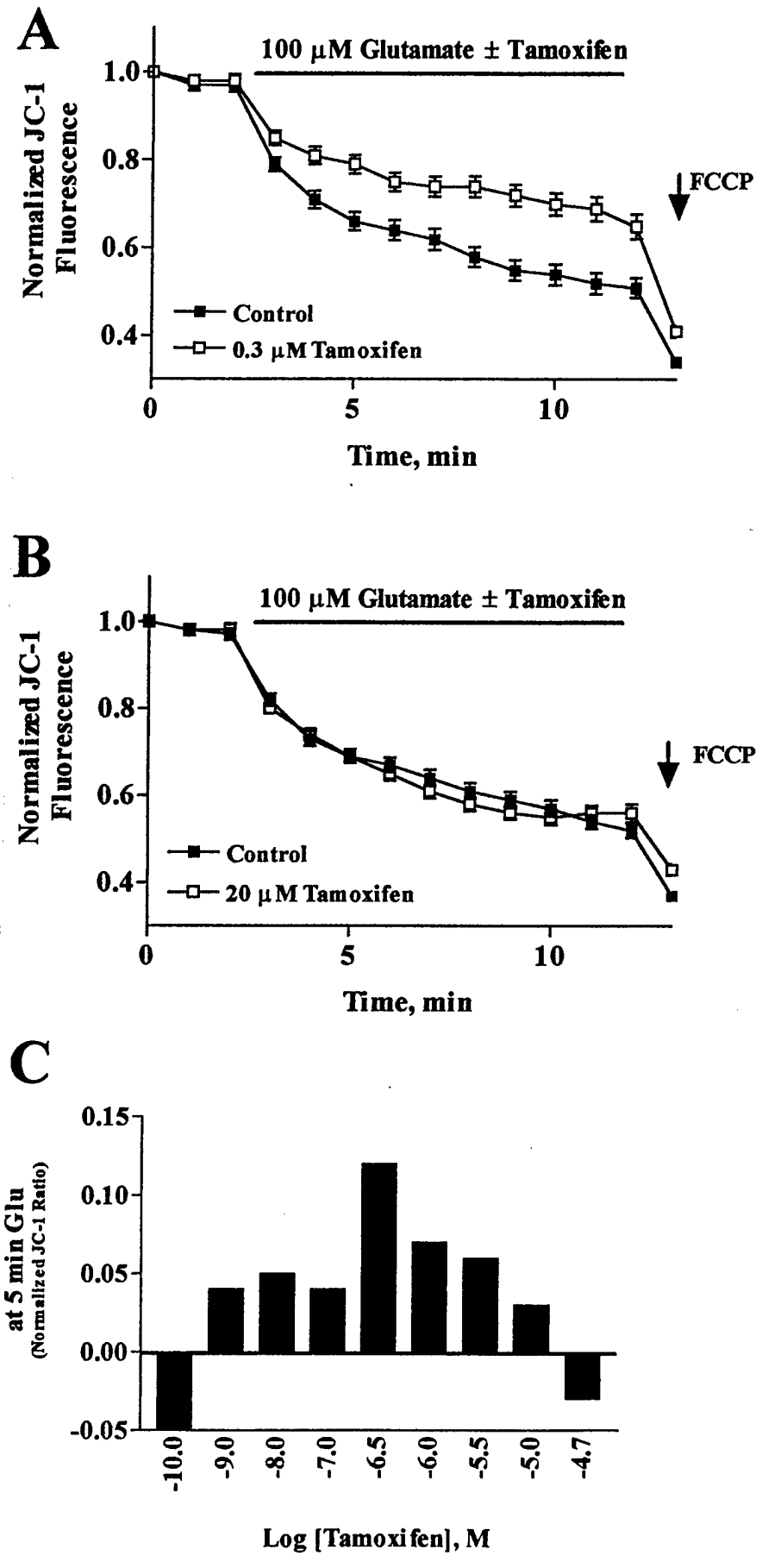

A
100 μM Glutamate ± Tamoxifen
Normalized JC-1 Fluorescence
1.0
0.8
0.6
0.4
FCCP
Control
0.3 μM Tamoxifen
0
5
10
Time, min
B
100 μM Glutamate ± Tamoxifen
Normalized JC-1 Fluorescence
1.0
0.8
0.6
0.4
FCCP
Control
20 μM Tamoxifen
0
5
10
Time, min
C
Difference From Control at 5 min Glu
(Normalized JC-1 Ratio)
0.15
0.10
0.05
0.00
-0.05
-10.0
-9.0
-8.0
-7.0
-6.5
-6.0
-5.5
-5.0
-4.7
Log [Tamoxifen], M

FIGURE 1

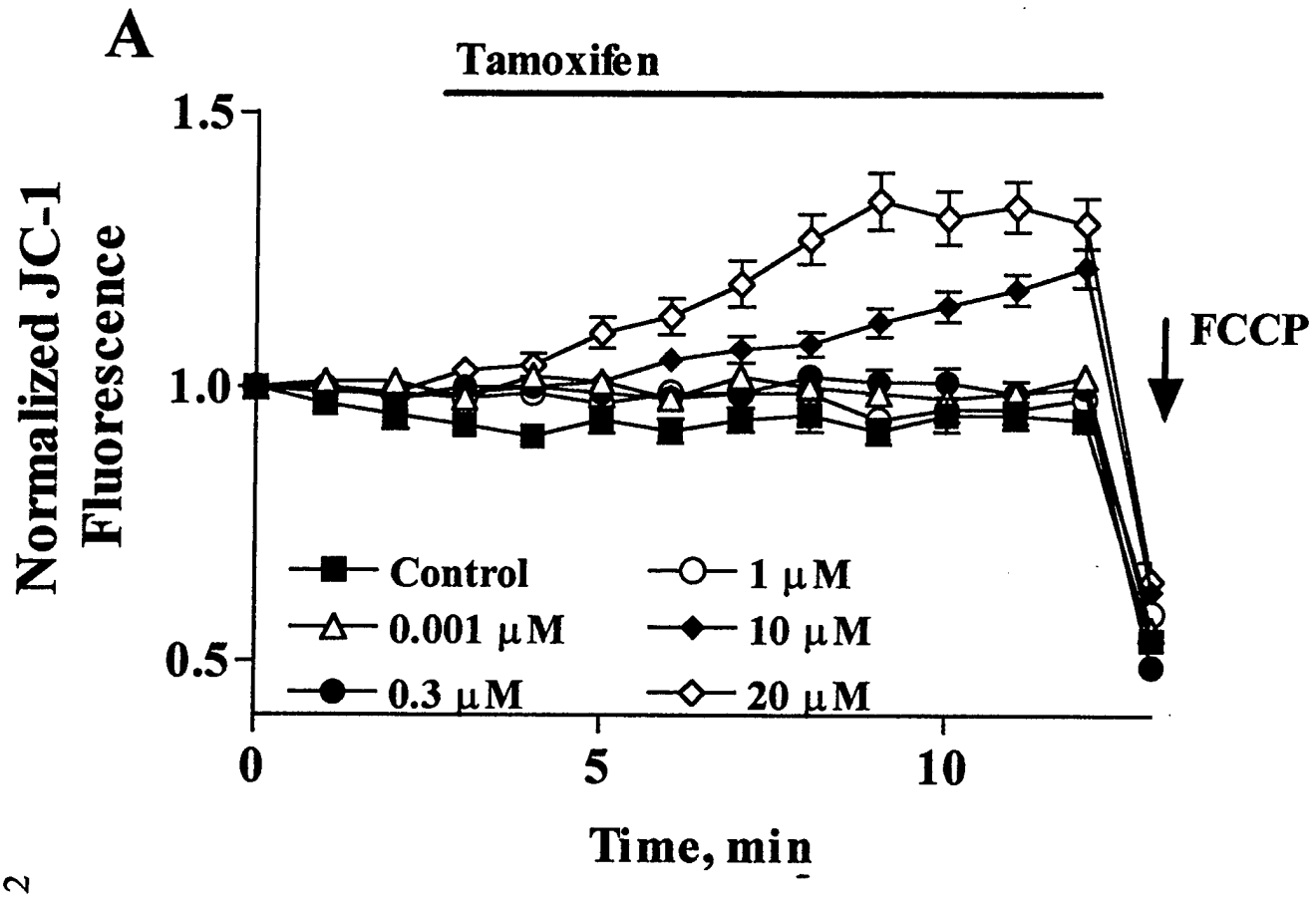

A
Tamoxifen
1.5
1.0
0.5
Normalized JC-1 Fluorescence
FCCP
Control
0.001 μM
0.3 μM
1 μM
10 μM
20 μM
0
5
10
Time, min

FIGURE 2

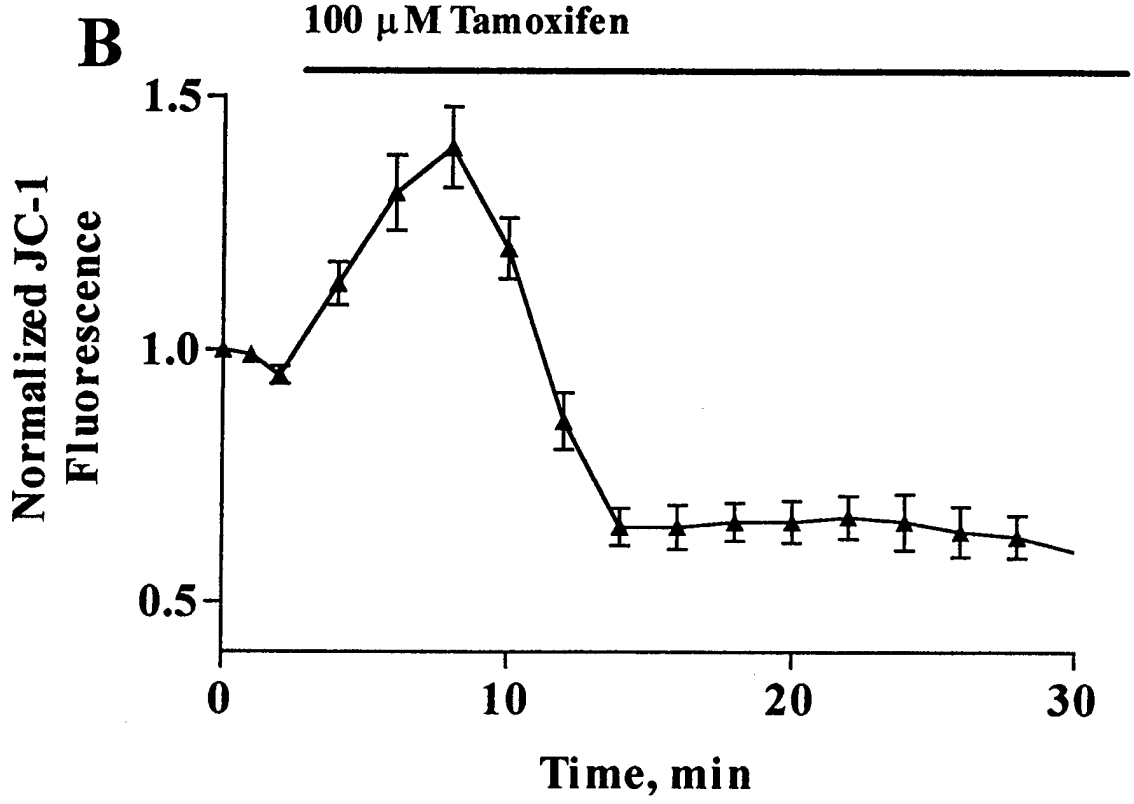
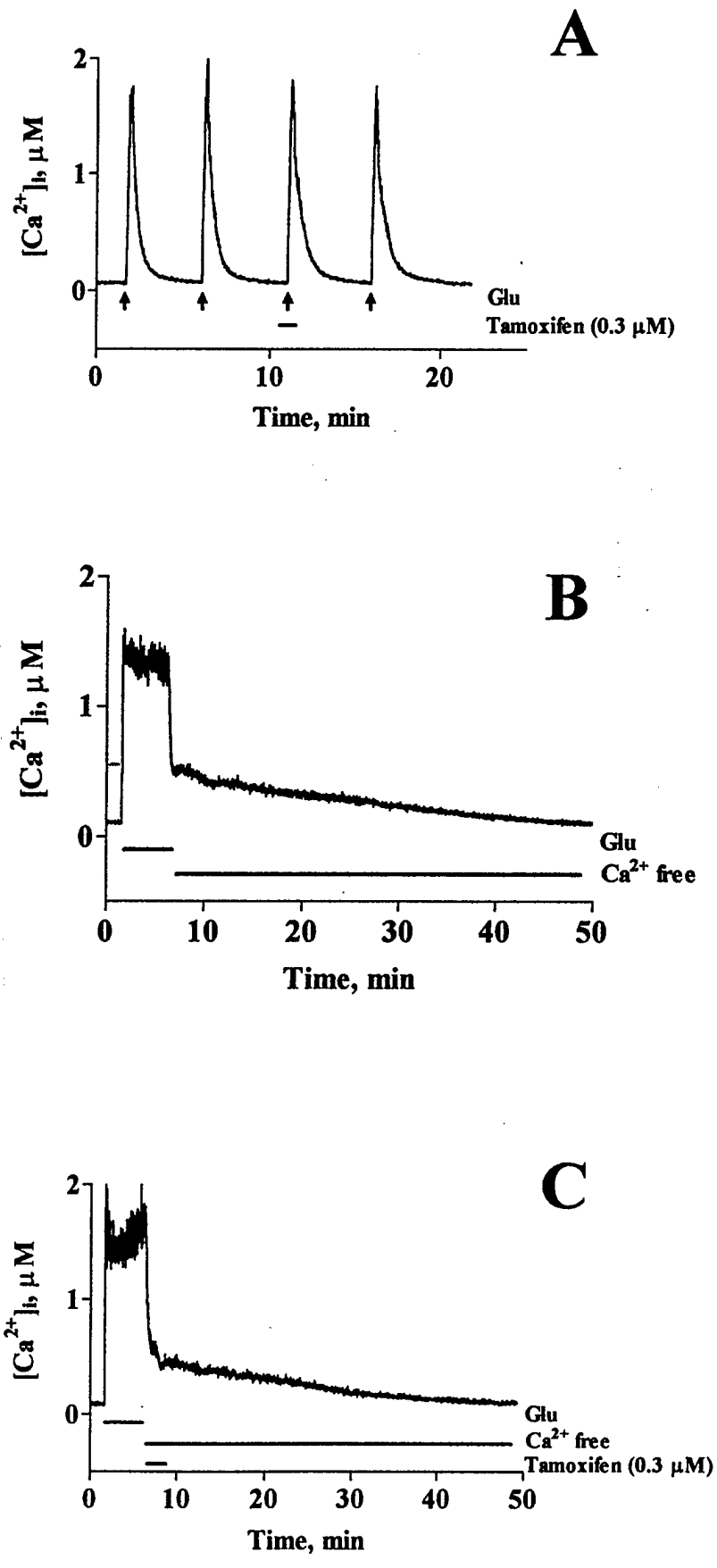

B
100 μM Tamoxifen
1.5
1.0
0.5
Normalized JC-1 Fluorescence
0
10
20
30
Time, min

FIGURE 3


A
$[Ca^{2+}]_i$, μM
2
1
0
Glu
Tamoxifen (0.3 μM)
0
10
20
Time, min
B
$[Ca^{2+}]_i$, μM
2
1
0
Glu
Ca^{2+} free
0
10
20
30
40
50
Time, min
C
$[Ca^{2+}]_i$, μM
2
1
0
Glu
Ca^{2+} free
Tamoxifen (0.3 μM)
0
10
20
30
40
50
Time, min

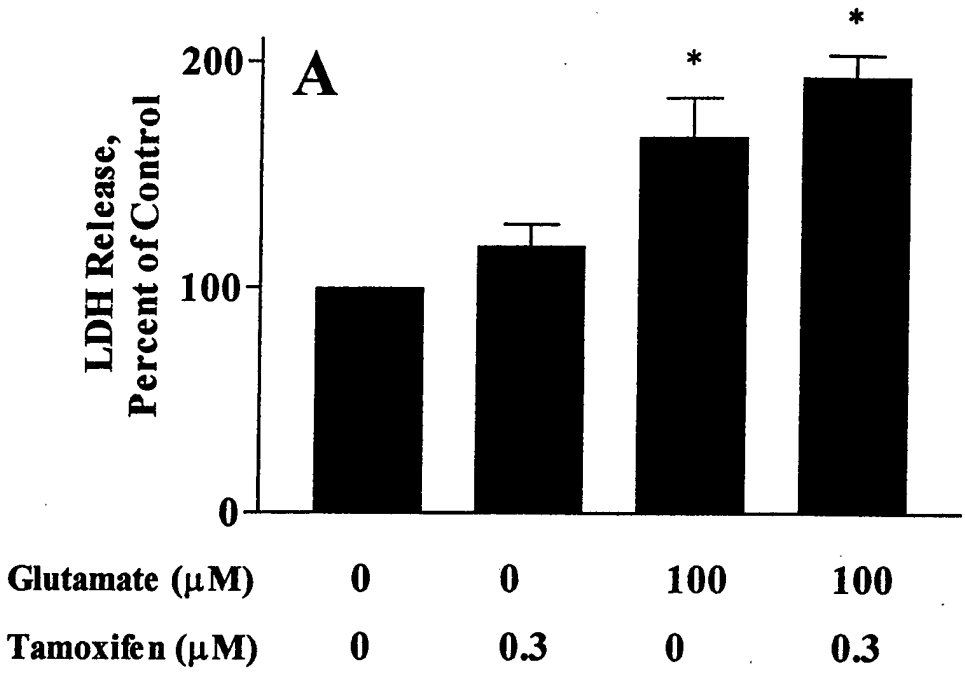

A
LDH Release, Percent of Control
200
100
0
*
*
Glutamate (μM) 0 0 100 100
Tamoxifen (μM) 0 0.3 0 0.3

FIGURE 4

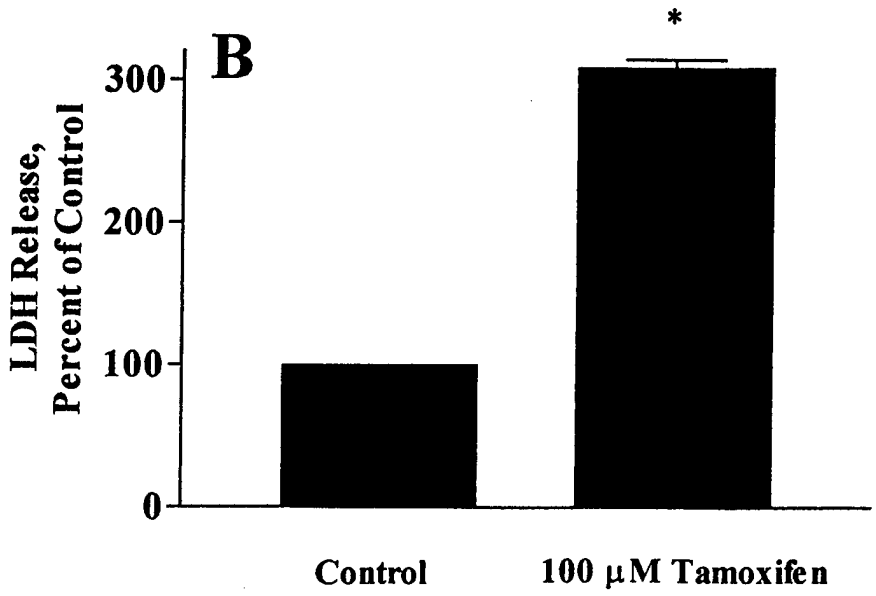
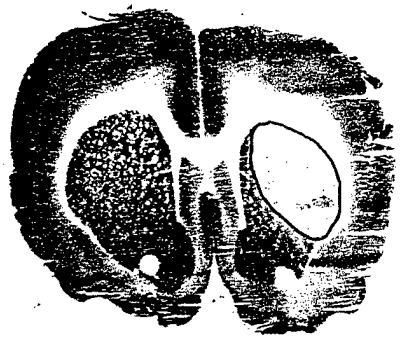

B
LDH Release, Percent of Control
300
200
100
0
*
Control
100 μM Tamoxifen

FIGURE 5

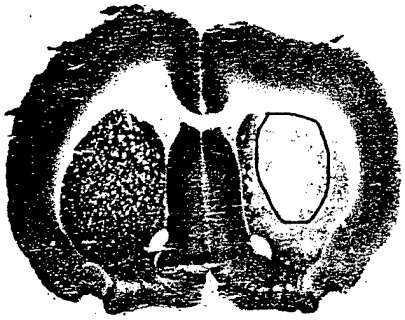
A

Control

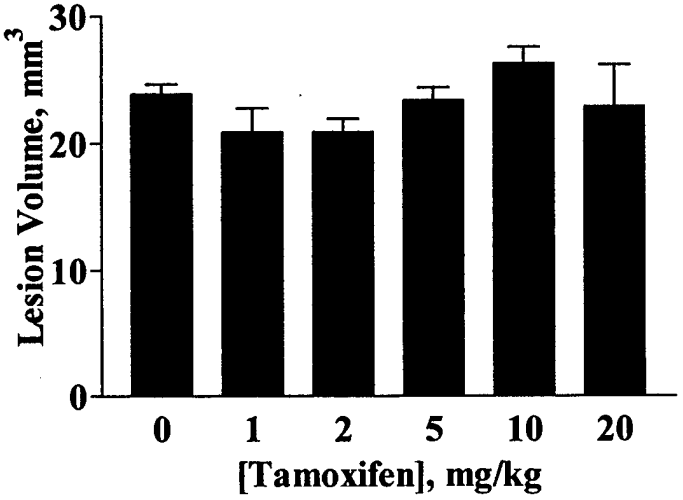


B

Tamoxifen (2 mg/kg)



C



853.3

EFFECT OF UBIQUINONE ANALOGUES ON GLUTAMATE-INDUCED INJURY IN RAT FOREBRAIN NEURONS J.M. Scanlon* and J.J. Reynolds. Dept. of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

An excitotoxic glutamate stimulus has various deleterious effects on mitochondrial function including depolarization of mitochondrial membrane potential ($\Delta\Psi$), production of reactive oxygen species (ROS), and possibly opening of the permeability transition pore (PTP). Ubiquinone is an integral part of the mitochondrial electron transport chain. Recent studies suggest that a ubiquinone analogue, coenzyme Q10 (CoQ10) is a putative enhancer of mitochondrial function and is neuroprotective in various models of neurodegeneration. In addition, some ubiquinone analogues can inhibit Ca^{2+}-dependent opening of the PTP (Fontaine *et al.*, 1998, JBC, 273:25734). The following ubiquinone compounds, ubiquinone-0 (UB0), ubiquinone-5 (UB5), decylubiquinone (decyl-UB), and ubiquinone-50 (CoQ10), were examined for their effects on neuronal survival, $\Delta\Psi$, and ROS production in the presence or absence of glutamate in primary cultures of rat forebrain neurons. Using confocal microscopic imaging ROS production was measured using the oxidation sensitive dyes, dichlorofluorescein (DCF) and dihydroethidium (DHE), and $\Delta\Psi$ was measured using the ratiometric dye JC-1. UB0 (50μM) was highly toxic producing almost 100% loss of trypan-blue excluding cells 24hrs after a 5min exposure. Decyl-UB (100μM) also reduced viability by 20%. UB5 (50μM) or CoQ10 (50μM) had similar effects on neuronal survival as controls (buffer alone). None of the ubiquinone compounds tested were able to protect against glutamate-induced neuronal death when given during and/or after glutamate exposure. In fact, glutamate-induced toxicity was enhanced by the addition of UB0 and UB5. The ubiquinone compounds had relatively no effect on DCF or DHE fluorescence with the exception of UB0 which increased DHE fluorescence. None of the compounds altered glutamate-induced production of ROS or the effects of an exogenous oxidant (hydrogen peroxide or xanthine/xanthine oxidase). UB0 (50μM) decreased the JC-1 ratio (aggregate/ monomer) by decreasing the aggregate signal in a reversible manner. UB0 also potentiated glutamate-induced mitochondrial depolarization. Decyl-UB (100μM) hyperpolarized $\Delta\Psi$ and inhibited glutamate-induced depolarization but was not neuroprotective. These data are not consistent with a predominant action of the ubiquinone analogues at the PTP.

Support Provided By: American Heart Association and USAMRMC, NS34138

853.4

ATRACTYLOSIDE MEDIATED MITOCHONDRIAL PERMEABILITY TRANSITION IN STRIATAL NEURONS.

G. Beutner[1], C.C. Alano[1], L. Lin, R.A. Gross[2], S. -S. Sheu[1]*

[1]Dept. of Pharmacology and Physiology, [2] Dept of Neurology, University of Rochester, SMD, Rochester, NY, 14642

Atractyloside (ATR) is a specific inhibitor of the c-conformation of the adenine nucleotide translocator (ANT) and a known inducer of the mitochondrial permeability transition pore (MPT). Inhibition of this non-specific channel is favored with ligands of the ANT stabilizing the m-conformation.

In isolated mitochondria from adult rat brain we observed a dose dependent swelling upon treatment with 50 to 300 μM ATR as measured by the decrease of absorbency at 540 nm. Mitochondrial swelling was prevented by pre-incubation with 200 nM N-metVal-cyclosporin (CyA). Incorporation of 0.5 to 5 μM ATR via lipofection into primary cultured striatal neurons from rat pups (E17) led to a dose dependent decrease of the intracellular ATP concentration to 80 % in 6 hours. Exposure of ATR treated neurons to 50 or 100 μM N-methyl-aspartic acid (NMDA) led to immediate swelling and necrotic cell death. In TMRE loaded neurons, a transient hyperpolarization followed by a strong depolarization approximately 90 minutes, was observed after adding ATR. The depolarization was prevented with CyA (200 nM). However, in neurons cultured with 1mM glucose and 2 mM pyruvate to enhance mitochondrial activity, a decrease in the mitochondrial membrane potential was observed after 30 minutes. In both sets of experiments we observed no further depolarization with CCCP. These results show that ANT might be the MPT and can be induced with ATR in cultured cells. ATR treatment enhances the sensitivity against NMDA. There is further evidence that the CyA/cyclophilin interaction may protect the binding site of the ANT for ATR.

Supported by NIH grant HL-33333 and CTR grant 4299R1.

853.5

AIT-082, A HYPOXANTHINE DERIVATIVE, PREVENTS MUCH OF THE DECREASE IN CEREBELLAR NEURON ATP FOLLOWING GLUTAMATE EXPOSURE. J.S. Bintner[2]*, M.P. Rathbone[1] and B.H.J. Juurlink[2]. [1]Dept. Medicine, Un. McMaster. [2]Dept. Anatomy & Cell Biology, Un. Saskatchewan, Saskatoon, SK, S7N 5E5, Canada.

Our previous experiments demonstrated that AIT-082, a hypoxanthine derivative, promoted neurite growth in cultured hippocampal neurons and protected these neurons from glutamate-induced toxicity. This suggested the possibility that AIT-082 promoted mitochondrial ATP production. To determine whether AIT-082 had any effect on neuronal mitochondria, two different experiments were performed. In the first, hippocampal neuron mitochondrial membrane potentials were examined using a rosamine derivative. Under control culture conditions the more distal the mitochondria were from the soma, the lower were their membrane potentials. AIT-082 (1.0 μM) resulted in increased membrane potentials in the more distal mitochondria. In the second experiment, cerebellar neurons were subjected to 100 μM glutamate and ATP levels were measured 30 min later. Glutamate addition caused a ~50% drop in cellular ATP. AIT-082 added 15 min following glutamate addition prevented much of this drop in cellular ATP. Our two observations suggest the possibility that AIT-082 may prevent mitochondrial uncoupling thus allowing more of the energy obtained from the the reducing equivalents entering the mitochondrial respiratory chain to be used for ATP production. *Supported by Neotherapeutics, Inc.*

853.6

MITOTRACKER DYES AS MARKERS OF MITOCHONDRIAL VOLUME JF Buckman*, SK Pal, GJ Kress and JJ Reynolds Dept. of Pharmacology, Univ. of Pittsburgh, Pittsburgh, PA 15261.

The majority of fluorescent dyes that measure changes in mitochondrial volume are also sensitive to changes in mitochondrial membrane potential ($\Delta\psi_m$). Reliable assessment of mitochondrial volume would be valuable for characterizing the permeability transition pore (PTP) independently of changes in $\Delta\psi_m$. Opening of the PTP is seen as a profound swelling of mitochondria, but a concurrent change in $\Delta\psi_m$ is typically observed, thus making it difficult to measure the morphological effects of permeability transition. MitoTracker dyes (Molecular Probes, Eugene, OR) are reportedly $\Delta\psi_m$-insensitive and label mitochondria by reacting with free sulfhydryls. We tested the effect of altered $\Delta\psi_m$ on loading and stability of the MitoTracker Green dye in primary forebrain neurons and astrocytes. We treated these cells with FCCP prior to, concomitantly or following loading with MitoTracker Green. Preliminary observations suggest that the fluorescence intensity of MitoTracker Green is more stable within astrocytes than neurons. Depolarization of mitochondria after loading results in an apparent diffusion of the dye at low concentrations (10-50nM) with increases in fluorescence intensity seen at higher concentrations (100nM – 1μM). However, when MitoTracker Green (500nM) and FCCP were co-loaded into cells, no changes in dye localization or fluorescence intensity were observed. Thus, MitoTracker Green can be reliably loaded into mitochondria regardless of $\Delta\psi_m$ but is sensitive to changes in $\Delta\psi_m$ after loading. MitoTracker Green may not be ideal for measuring changes in mitochondrial volume resulting from permeability transition. MitoTrackers Red and Orange are being investigated.

Supported by NS34138, USAMRMC and the American Heart Assoc.

853.7

MITOCHONDRIAL-SIGNALING FOR APOPTOSIS FOLLOWING NMDA RECEPTOR ACTIVATION IS RELATED TO AN ENERGY CRISIS. Samantha L. Budd* & Stuart A. Lipton. CNS Research Institute, Brigham & Women's Hospital and Harvard Medical School, Boston, MA 02115.

Mitochondria play an essential role in the survival of cells. In neurons, the continual restoration of ion gradients by ATP consuming pumps requires robust mitochondria. In addition to ATP synthesis, mitochondria accumulate Ca^{2+}, generate superoxide, regulate matrix enzymes, and release apoptotic signaling molecules. Most mitochondrial functions are linked to the mitochondrial membrane potential ($\Delta\psi_m$), the driving force behind ATP synthesis. It is thought that complete bioenergetic collapse leads to necrosis, while some ATP is required for apoptosis. However, a moderate energetic restriction in neurons may precipitate apoptosis. Depending on the intensity of an excitotoxic insult, cerebrocortical neurons in mixed cultures are susceptible to death via necrosis or apoptosis. Here necrosis was produced after a relatively prolonged excitotoxic insult (e.g., 2 mM NMDA for 30-60 min), but apoptosis after a shorter exposure (300 μM NMDA for 20 min). NMDA receptor (NMDAR)-mediated neuronal apoptosis was accompanied by Ca^{2+}-induced $\Delta\psi_m$ depolarization, transient energy (ATP) loss, caspase-3 activation, and release of cytochrome c. Specific inhibition of the mitochondrial adenine nucleotide translocator (ANT) with bongkrekic acid (BA) reduced NMDAR-mediated apoptosis. In addition, BA prevented NMDAR-induced $\Delta\psi_m$ depolarization, promoted recovery of cellular ATP content, and ameliorated caspase-3 activation. However, the presence of BA during NMDAR activation did not prevent the movement of cytochrome c into the cytoplasm. These results indicate that release of cytochrome c alone was not sufficient to induce neuronal apoptosis. BA, by inhibiting the ANT during NMDAR activation, obstructed the release of nucleotides during a critical period. In contrast, staurosporine-induced apoptosis was not associated with $\Delta\psi_m$ depolarization or ATP loss, and BA was ineffective in preventing staurosporine-induced apoptosis or activation of caspase-3. Staurosporine also stimulated the release of cytochrome c, indicating that $\Delta\psi_m$ depolarization was not obligatory for its release. Thus in cerebrocortical cultures excessive NMDAR activation, but not staurosporine, precipitates neuronal apoptosis via mitochondrial dysfunction. Supported by the Wellcome Trust and NIH P01 HD29587.

853.8

SELECTIVE VULNERABILITY OF MOTOR NEURONS TO AMPA/KAINATE RECEPTOR-MEDIATED INJURY AND MUTANT Cu/Zn SUPEROXIDE DISMUTASE. S.S. Rao[1]*, S.G. Carriedo[3], J. Nguyen[1] and J.H. Weiss[1,2,3]. Depts of [1]Neurology; [2]Anatomy and Neurobiology; [3]Neurobiology and Behavior, U.C. Irvine, Irvine CA 92697-4292, USA.

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterized by the selective loss of upper and lower motor neurons (MN). The reason for the high vulnerability of MNs in ALS is poorly understood. One clue derives from findings suggesting a role for excitotoxic injury in the disease. Our observation that cultured MNs (identified by the neurofilament antibody SMI-32) generally express Ca^{2+} permeable AMPA/Kainate (Ca-A/K) channels provides a feature that may underlie their demonstrated selective vulnerability to AMPA/kainate receptor-mediated injury. Excitotoxic activation of Ca-A/K channels leads to large rises in intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) mitochondrial membrane depolarization (loss of $\Delta\Psi_m$) and increased reactive oxygen species (ROS) production. Indeed, MN injury resulting from these exposures is Ca^{2+} -dependent and can be attenuated by antioxidants. Another clue arises from studies demonstrating that expression of mutant forms of Cu/Zn superoxide dismutase (SOD-1) found in familial ALS patients, induce an ALS-like MN degeneration in transgenic mice. Mechanisms through which mutant SOD-1 leads to selective MN loss are poorly understood. Present studies set out to characterize the properties of MNs expressing mutant SOD-1 with the aim of elucidating potential interactions and synergism between effects of SOD-1 mutations and MN injury caused by Ca^{2+} influx through Ca-A/K channels. We have prepared dissociated spinal cord cultures using hemizygous SOD-1 (G93A) transgenic mice (cultures from littermates lacking the mutant gene are used as controls). Early characterization demonstrates similar morphology of putative MNs (assessed by SMI-32 staining) between transgenic and control cultures. Ongoing studies are seeking to compare the vulnerabilities transgenic and control MNs to AMPA/kainate toxicity and to examine effects of the G93A mutant on Ca-A/K channel expression. Further studies are using fluorescent imaging techniques to compare AMPA or kainate induced changes in $[Ca^{2+}]i$, $\Delta\Psi_m$ and ROS generation. These studies may provide new insights into the basis of the selective MN loss in ALS. *Supported by NIH grants NS30884 and AG00836 (JHW) and an ALS Association research grant (JHW).*